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Brit. J. Pharmacol. (1954), **9**, 1.

INVESTIGATION OF THE TOXICITY OF A SERIES OF DEXTRAN SULPHATES OF VARYING MOLECULAR WEIGHT

BY

KENNETH W. WALTON

From the Department of Experimental Pathology, University of Birmingham

(RECEIVED MAY 5, 1953)

In previous communications (Walton, 1952, 1953) it was reported that differences of behaviour were demonstrable *in vitro* between members of a series of dextran sulphates of varying molecular weight. Although all the compounds examined showed heparin-like anticoagulant activity, those with molecular weights in excess of 40,000 differed from heparin and from the dextran sulphates of smaller molecular weight in precipitating the plasma proteins, and especially fibrinogen, in causing agglutination of the platelets and leucocytes and in producing alterations in the sedimentation rate of the erythrocytes.

Grönwall, Ingelman, and Mosimann (1945), using a more limited series of molecular sizes, showed that the toxicity of dextran sulphates for experimental animals declined as the molecular weight decreased. When the *in vivo* behaviour of the present series was investigated, it was found that the compounds which precipitated plasma-protein and agglutinated blood-cells *in vitro* were toxic in the intact animal. A direct correlation was observed between the molecular weight of the dextran sulphate and the degree of toxicity (Walton, 1951). This observation has since been confirmed by Stavely, Baker, and Payne (1952).

The present communication gives the results of an investigation into the nature and mechanism of this toxicity. The findings are broadly in agreement with those of Piper (1945b) and of Astrup and Piper (1946), who examined the sulphuric esters of several other polysaccharides (cellulose, starch, chitin, etc.).

Dextran sulphate of low molecular weight, which did not interfere with the stability of the plasma proteins or of the blood-cells, was found to have a toxicity comparable with that of commercial heparin when administered to experimental animals. The preliminary therapeutic trial of one such compound has been reported separately (Ricketts, Walton, van Leuven, Birbeck, Brown, Kennedy, and Burt, 1953).

MATERIALS AND METHODS

Dextran Sulphates.—The compounds used were those previously described (Walton, 1953). They were selected members of a series prepared by Ricketts (1952a). The average molecular weight of the D size compounds was 458,000; of the E size, 47,000; and of the I size, 7,500.

Heparin.—The anticoagulant activity of the dextran sulphates was assayed by the method of Kuizenga, Nelson, and Cartland (1943) against a sample of International Standard Heparin. Commercial heparin preparations were used for qualitative comparisons with the dextran sulphates. For most purposes, the preparation used was "Pularin" (Evans), batches No. F 85020 and No. N 10090, both assaying at 90 i.u./mg. In one specified experiment, the following additional preparations were employed: (*a*) British: "Liquemin" (Roche), batch dated August 31, 1948, 122 i.u./mg.; Heparin (Boots) batch No. 3583, 102 i.u./mg.; Heparin (British Drug Houses) batch No. 30607/c, 160 i.u./mg. (*b*) American: Heparin (Lederle) batch No. NP 115–128, 100 i.u./mg. (*c*) Swedish: Heparin "Leo" (no batch number or date supplied), 100 i.u./mg.

Platelet-counting Methods.—Lempert's (1935) modification of Kristenson's (1922) method, and the method of Rees and Ecker (1923), modified by increasing the concentration of formalin to 1%, were used. Since high concentrations of heparin and the dextran sulphates formed insoluble precipitates with cresyl blue, unstained platelets were counted in some experiments with the phase-contrast microscope.

Clotting-time Methods.—In experiments on small animals, necessitating frequently repeated estimations of the clotting-time, the method of Dale and Laidlaw (1912) was employed using standardized capillary-tubes containing steel shot of uniform size. Otherwise the method of Lee and White (1913) was used. All estimations were performed at 37° C.

Histological Techniques

Fixatives.—10% neutral formol-saline, 80% alcohol and Heidenhain's "Susa" were employed as routine fixatives. A preliminary trial of 4% basic lead acetate (Holmgren, 1940) for the demonstration of metachromatic material in reticulo-endothelial cells showed no substantial improvement over these.

Staining Methods and Materials.—In addition to routine staining of all sections with Erlich's haematoxylin and eosin, the following were used :

Metachromatic Stains.—Batches 851, 948 and 1234 of Toluidine Blue G were supplied by G. T. Gurr, Ltd. Batch No. 005804 of Azure A and batch No. 5146 of Thionine were supplied by Hopkin and Williams, Ltd. All these stains gave satisfactory results with Hughesdon's (1949) technique. Mallory's phloxine-methylene blue stain, containing Azur II (Lillie, 1948), also gave satisfactory results.

Fibrin Stains.—The recognized "fibrin" stains were found not to distinguish between fibrin and fibrinogen. The methods used were: the Weigert-Gram technique ; Mallory's phosphotungstic acid haematoxylin ; phloxine-tartrazine (Lendrum, 1947) ; and the acid picro-Mallory method (Lendrum, 1949).

Other Stains.—Haematoxylin-Giemsa and the Turnbull-Jenner (Turnbull, 1931) methods were used for the identification of intravascular leucocytes and platelets. The periodic acid Schiff technique (McManus, 1946 ; Hotchkiss, 1948) was employed at first, but was found to give weakly positive staining reactions with dextran sulphate and fibrinogen, singly and in combination.

Animals.—Albino mice (20–45 g.), albino rats (150–300 g.), guinea-pigs (450–700 g.), and rabbits (2–4 kg.) were used. All these animals were kept on standard pellet diets during the course of the experiments.

Results

The dextran sulphates were found to be effective anticoagulants by parenteral administration only. No evidence of absorption or of toxic effects was obtained on oral or sublingual administration. Oral and sublingual heparin is similarly ineffective according to most workers (see Kennedy and Brown, 1952 ; McDevitt, Huebner, and Wright, 1952 ; and Tomich and Woollett, 1952).

Effective Dose.—As with heparin, some variation of response to intravenous dosage was encountered amongst animals of the same species, but, in general, it was concluded that a single injection of 6–8 mg./kg. body-weight was sufficient to prolong the clotting-time to about three times the normal, when estimated one hour after injection (Walton, 1951). The administration of similar doses subcutaneously, intramuscularly or intraperitoneally was also effective, but absorption was slower and more uneven, and the effect obtained less reproducible, than when the material was given intravenously.

Equivalent doses of this order of the various dextran sulphates and of heparin were given intravenously to rabbits and their effects upon the plasma proteins and the blood cells compared (Figs. 1 and 2).

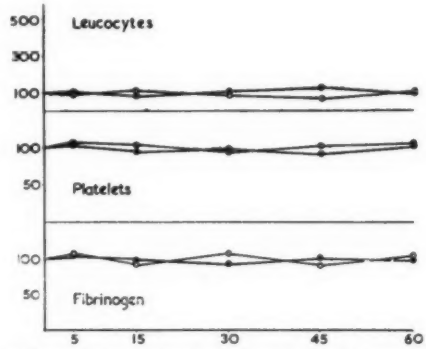


FIG. 1.—Effect of the minimal effective dose of heparin (1 mg. kg. body-weight) and of the I size dextran sulphate (6 mg. kg. body-weight) on the levels of the leucocytes, platelets and plasma fibrinogen in the rabbit. Ordinate: percentage deviation from pre-treatment level. Abscissa: min. after intravenous injection. ●——●: Heparin. O——O: I size dextran sulphate.

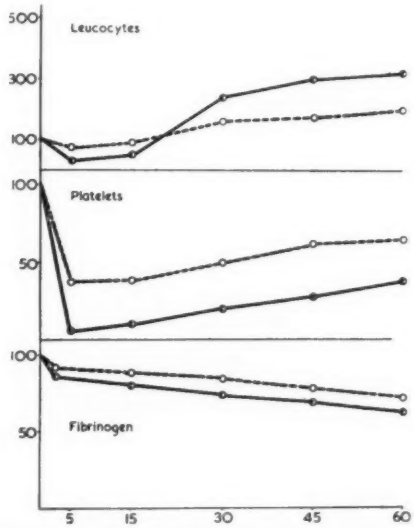


FIG. 2.—Effect of 6 mg./kg. body-weight of the E and D sizes of dextran sulphate on the levels of the leucocytes, platelets and plasma fibrinogen in the rabbit (for comparison with Fig. 1). Ordinate: percentage deviation from pre-treatment level. Abscissa: min. after intravenous injection. ◐——◐: D size dextran sulphate. O——O: E size dextran sulphate.

It will be seen from Fig. 1 that heparin and the small molecular weight dextran sulphate produced little or no alteration of the plasma fibrinogen or of the numbers of the formed elements of the blood. On the other hand, as shown in Fig. 2, the D and E size dextran sulphates produced: (*a*) A

steady fall of the plasma fibrinogen during the first hour. This was followed by a slow restoration to the normal level during the next two hours. (*b*) A sharp fall in the number of single platelets, with the appearance of large numbers of clumped platelets. These platelet-clumps varied in size, making accurate counts difficult. They showed the same general characters as those observed *in vitro* (Walton, 1953), individual platelets being enmeshed in amorphous material stained lilac in colour by the cresyl blue present in the diluting fluid. Small shreds of similar material free from adherent platelets were also present. (*c*) An equally abrupt drop in the number of leucocytes. In contrast with the behaviour of the platelet-count, however, the white-cell count was restored to normal within half an hour and often continued to rise subsequently to produce a moderate leucocytosis. (*d*) A slight and inconstant fall of the haemoglobin level. When animals showing this last phenomenon were more closely investigated, the red-cell count was found to fall proportionately and no evidence was obtained of haemolysis. Animals repeatedly injected with these compounds showed a simple normochromic anaemia with a persistently high reticulocyte count. Such animals were usually found to have one or more bleeding-points at autopsy (see below) and this was held to account for the changes inconstantly observed.

The effect upon the platelets, *in vitro* and *in vivo*, of the large molecular weight dextran sulphates, is closely similar to that shown by the cellulose, starch, and chitin sulphate esters previously investigated by Piper (1945a). Because of the eventual restoration to normal numbers and the disappearance of platelet-clumps, this author regarded the *in vivo* phenomenon as a reversible one in which the platelet-clumps could dissociate again into their individual components. No details of parallel investigations of the effect upon the leucocytes were given.

The present finding of a concomitant fall in leucocytes which is succeeded by leucocytosis strongly suggests that the thrombocytopenia and leucopenia were both corrected by the same mechanism, namely, fresh output of these elements by the marrow. This contention is supported indirectly by the work of Stetson and Good (1951), who found a similar fall of platelets and leucocytes following the administration of glycogen, agar, acacia, and other macromolecular colloids to animals sensitized to unrelated antigenic materials. These authors showed that the return to normal numbers of the platelets and leucocytes did not occur in animals in which the marrow had been rendered aplastic by nitrogen mustard.

Other evidence that the effect on the platelets was irreversible was provided by the finding that platelet thrombi were demonstrable in various organs long after the effect on the clotting-time of a single injection of the large molecular weight dextran sulphates had worn off.

Acute Toxicity of the Dextran Sulphates

In pilot experiments an investigation was made of the relation between these effects upon the blood-cells and plasma-proteins and the toxic manifestations produced by very large doses of the large molecular weight compounds. Small groups of mice and rats were given single intravenous or intraperitoneal injections of doses varying between 2 g./kg. and 100 mg./kg. Animals dying within one week were autopsied as soon as possible after death. Survivors were killed at varying intervals up to 28 days after the injection and autopsied similarly. As a result it was concluded that although the immediate cause of death varied with the level of dosage employed, the mechanism of production of the lesions found was always related directly to the effects described above.

It should be noted that with these massive doses of the anticoagulants, blood taken from the animals within an hour of the injection remained uncoagulated for 24 hours or longer and that, in surviving animals, the clotting-time was elevated —frequently to four or five times its normal value —even on the day following the injection.

Pathology

Group 1. Animals Dying Within a Few Minutes of Injection

Some animals given doses in excess of 500 mg./kg. of the D size compound, or doses in excess of 900 mg./kg. of the E size compound, died almost immediately, showing marked respiratory and circulatory embarrassment, often attended by convulsions.

At autopsy, the macroscopic findings in these animals were strikingly few, consisting merely of generalized hyperaemia and venous congestion of the thoracic and abdominal organs, with scattered petechial and purpuric haemorrhages beneath the serosal surfaces. The heart invariably showed dilatation of the right auricle and ventricle, the left chambers being relatively normal.

Histological examination of sections stained with Ehrlich's haematoxylin and eosin showed widespread embolism of vessels of varying calibre by pinkish granular or hyaline material associated with clumps of leucocytes. The process was demonstrable with greatest intensity in the lung, but was also demonstrable in other organs. In sections from material fixed and stained appropriately, clumps of platelets could also be identified. These

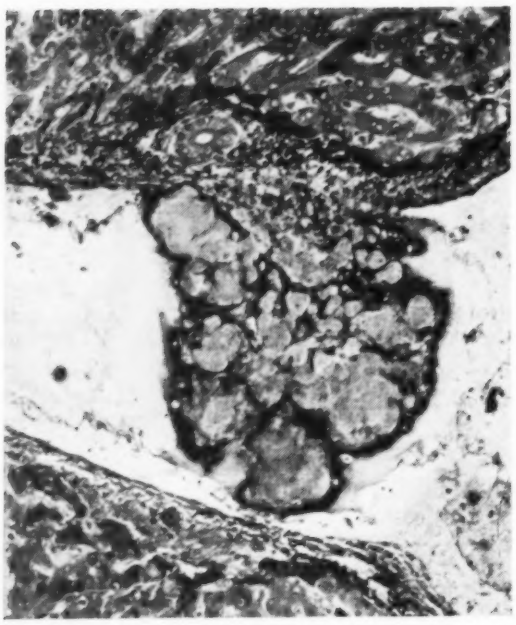


FIG. 3.—Portion of a large portal vein in the liver showing a platelet mass adherent to the wall. Note that the agglutinated platelets are enmeshed in a network of material giving a positive staining reaction for fibrin or fibrinogen. The platelet mass is being infiltrated at its point of attachment by cells derived from the sub-intimal tissues. Rabbit given 500 mg./kg. body-weight of the D size dextran sulphate. Lendrum's acid picro-Mallory method. × 228.

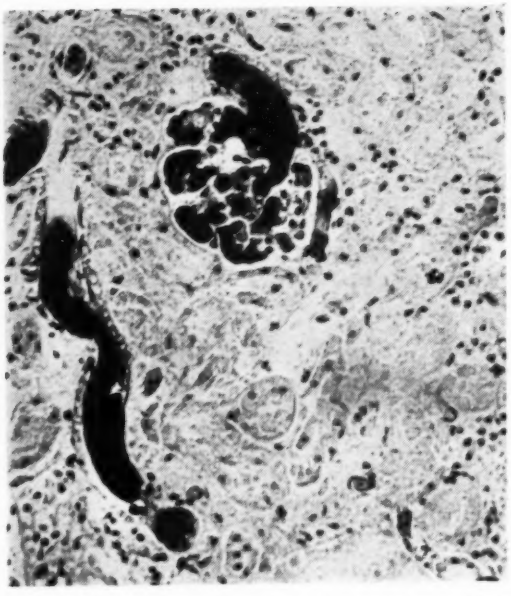


FIG. 5.—Embolism of renal arterioles and glomerular capillaries by metachromatic material. Rabbit given 100 mg./kg. dextran sulphate E. Toluidine blue. × 220.

FIG. 4.—Embolism of pulmonary arterioles and capillaries by metachromatic material. Rat given 500 mg./kg. body-weight E size dextran sulphate. Toluidine blue. × 320.

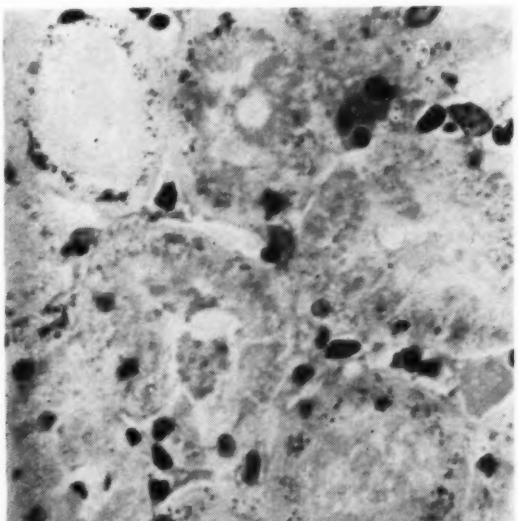


FIG. 6.—Rat kidney. The epithelium of the proximal convoluted tubules contains small granules of metachromatic material following a dose of 150 mg./kg. of the E size dextran sulphate. Toluidine blue. × 700.

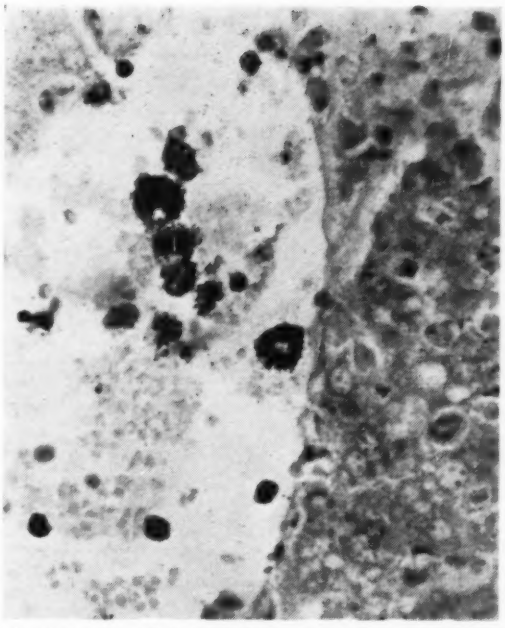


FIG. 7.—Large centrilobular vein in the liver of a mouse given 400 mg./kg. of the D size dextran sulphate. Many free phagocytic cells in the lumen of the vessel contain granular metachromatic material. Toluidine blue. × 400.

conglutination thrombi were apparently free in the lumen of a vessel, but serial sectioning frequently showed that they were adherent at some point to the endothelial surface of the vessel (Fig. 3). Sections stained with toluidine blue showed the granular material surrounding the formed elements to be strongly metachromatic—i.e., to show the same staining reaction as that given by the dextran sulphate-fibrinogen complex *in vitro* (Walton, 1953). By this means, it was possible to see that in the lung the whole vascular tree, down to the alveolar capillaries, was filled with the material (Fig. 4). Vessels of varying calibre in the kidney also contained similar material and the effect was particularly striking in the glomerular capillaries (Fig. 5). The portal veins and sinusoids in the liver contained material giving similar staining reactions, and arterioles and capillaries in the interstitial tissue of the ventricular myocardium were affected. Complete blockage of the main coronary arteries or of the primary subdivisions of the pulmonary artery was never encountered though it was looked for repeatedly. Embolism of small myocardial vessels was accompanied by patchy areas of coagulative necrosis of myocardial fibres.

Apart from the amorphous metachromatic material present in the lumina of vessels, it was noted that granules of metachromatic material were present in occasional large mononuclear cells in the blood and that similar material was present in reticulo-endothelial cells in various situations. Thus, the Kuppfer cells in the liver sinusoids, littoral cells in the splenic pulp and large mononuclear cells in the femoral bone-marrow were affected in this manner (Figs. 10 and 11). In animals injected intravenously, only occasional cells giving a similar staining reaction were observed in lymph-nodes in various parts of the body, but the abdominal lymph-nodes were found to be heavily infiltrated with these cells in animals given the material intraperitoneally. With one exception, no metachromatic material was demonstrable in the parenchymal cells of any of the organs examined. This exception was the kidney, where in rats, but not in mice, granules of metachromatic material were demonstrable in the cells of the proximal convoluted tubules (Fig. 6).

Mast-cells, in the normal rat and mouse, give a metachromatic staining reaction with toluidine blue, but are situated quite differently from the cells giving the metachromatic reaction at the sites described above. Mast-cells are distributed, for the most part, in the delicate interstitial tissue beneath the serosal surfaces of organs or in the connective tissues surrounding bronchi and other ducts, vessels, and in the portal tracts. This was verified by the examination of tissues from uninjected control animals. The only situation where confusion was likely to arise was in the bone-marrow, and here the very large numbers of metachromatic cells in treated, as opposed to untreated, animals rendered the distinction unnecessary. Basophilic leucocytes were rarely seen in the blood of controls, so, again, the marked increase in numbers of large cells containing metachromatic material in the peripheral blood was thought to be significant (Fig. 7).

Group 2. Animals Dying 1–4 Hours After Injection

Other animals given doses of the same order as Group 1 showed similar acute symptoms and signs, but recovered. A proportion of such animals died one to four hours later.

At autopsy, the most striking feature in this group of animals was an acute haemorrhagic pulmonary oedema. Frothy, blood-stained fluid could be seen to be escaping from the external nares, and the larynx, trachea and main bronchi were filled with similar fluid. The lungs were voluminous, and, on section, exuded quantities of this fluid. Numerous blotchy haemorrhagic areas were seen beneath the visceral pleura. Rarely, these showed a central portion raised above the cut surface and resembling an infarct, but diffuse haemorrhage usually extended for a variable distance into the lung parenchyma. Sometimes a

complete lobe or the whole lung was thus involved. The heart showed dilatation of the right side similar to that observed in the previous group of animals. The abdominal organs showed an intense generalized congestion. Occasional petechial and small purpuric haemorrhages were observed beneath the serosal surfaces of the stomach and intestines. The liver was congested and showed accentuation of its lobular pattern. Occasional punctate haemorrhages were present in the cortex of the markedly congested kidneys. These haemorrhages were sometimes sufficiently numerous to simulate the appearance of the " flea-bitten " kidney of human embolic nephritis.

Histological examination of the tissues from these animals confirmed the acute haemorrhagic pulmonary oedema. Conglutination thrombi or emboli were again visible in the pulmonary vessels. In some fields there was intense intra-alveolar haemorrhage, while elsewhere the exudate present in the alveoli was faintly eosinophilic, granular, protein-like material. In the centres of the haemorrhagic areas it was sometimes possible to distinguish areas of definite infarction. Small areas of haemorrhagic necrosis were also found in the liver, and minute necrotic foci were present in the spleen. The latter commonly involved the Malpighian follicles, but occasionally similar areas were also present in the pulp. The accumulation of metachromatic material in the reticulo-endothelial cells of the liver, spleen and bone-marrow was much more pronounced. The granules of metachromatic material in the cells of the proximal convoluted tubules of the kidneys of rats were scanty in numbers in this group of animals.

Group 3. Animals Dying 12 Hours to 7 Days After Injection

The remaining animals given doses similar to those of Group 1 were obviously gravely ill. Most of them succumbed within 24–48 hours, but occasional animals survived up to four or five days. Some of the animals given doses of the D size compound between 500 mg./kg. and 200 mg./kg., or of the E size compound between 900 mg./kg. and 300 mg./kg., also died within this period, but the numbers of survivors increased as the size of the dose decreased. Only an occasional animal died within one week when given doses of the D size compound of 100 mg./kg. or less, or of the E size compound of 300 mg./kg. or less. The fatalities at these various dosages showed lesions of similar character and will be described together.

Post-mortem examination of these animals showed that the cause of death was always haemorrhage. The site and source of this haemorrhage varied. Many animals again showed evidence of severe intrapulmonary bleeding. This was often so extensive as to involve a whole lobe or lung. Sometimes a central area of infarction, which was now more easily distinguishable, was surrounded by diffuse and irregular haemorrhage into the adjacent parenchyma. Other animals showed intraperitoneal bleeding. This was observed only occasionally in animals injected intravenously (via the tail vein) but very commonly in animals injected intraperitoneally. Other sites of intra-abdominal bleeding were the testicular omentum, the retroperitoneal tissues and the mesentery. Small petechiae were seen frequently beneath the peritoneum of the anterior abdominal wall ; in the subserous tissues on the surface of the stomach, caecum and large intestine ; beneath the visceral pericardium and pleura and occasionally scattered at random in the subcutaneous fascia. Melaena was rarely encountered. In the few animals in which it occurred, a bleeding point was seldom demonstrable in the alimentary tract, but in two animals it was associated with a haemorrhagic necrosis, due to infarction, of the duodenum. The abdominal organs were blanched and soft in consistency. The liver showed blurring of its normal pattern with small yellowish areas scattered at random through its substance. Obvious areas of infarction were present in the spleen. The kidneys showed many pin-point haemorrhages against the pale, swollen background of the cortex. Occasionally obvious areas of cortical necrosis were present. Urine collected from the bladder and centrifuged showed the presence of red-cells, pus-cells, amorphous debris and occasional granular casts.

Histologically, it was possible only occasionally to define with precision the point from which the haemorrhage had originated. The haemorrhage was usually so extensive as to obliterate pathological changes in the area. In a few instances, where the haemorrhage had been less severe, it was possible to trace the bleeding to an area showing micro-embolism and infarction. It was therefore assumed that the larger lesions had originated from involvement of larger vessels in a similar manner, and occasionally this assumption was supported by the finding of an arteriole plugged with amorphous metachromatic material, in the affected area.

In the animals surviving longest in this group, there was evidence of siderotic phagocytic activity in the lung, liver and spleen, and of the usual reactive changes at the margins of areas of necrosis in the liver, spleen and other organs.

The reticulo-endothelial cells of the liver, spleen and bone-marrow were still heavily loaded with

metachromatic material. The accumulation of metachromatic granules in the epithelium of the proximal convoluted tubules in the rat was maximal in this group of animals.

Group 4. Surviving Animals

When these animals were killed occasional small haemorrhages and haematomata were found in similar situations to those seen in the previous group, but these lesions were less severe in extent and fewer in number.

Histological examination showed hyaline material present in vessels attached to the wall and becoming endothelialized in the animals which had survived longest. This was seen most commonly in the pulmonary vessels, but was also found in the vessels of the spleen and kidney. Granular metachromatic material was still present in abundance in the reticulo-endothelial cells of the liver, spleen and marrow.

To summarize, parenteral administration of doses between 2 g./kg. and 100 mg./kg. of the large molecular weight compounds caused death either immediately, by embolism (mainly pulmonary); after some delay, by acute pulmonary oedema; or more remotely, by infarction and haemorrhage, depending upon the level of dosage employed. The toxicity of the E size compound was less marked than that of the D size compound. The doses used in this experiment were vastly in excess of the average effective dose. But surviving animals, given the smaller doses, showed lesser degrees of the process producing fatalities. For this reason, in a second experiment, the range of doses used was designed to cover the possible "therapeutic" range of the less toxic compound.

A group of twenty rats was given the E size dextran sulphate in doses varying between 80 mg./kg. and 5 mg./kg. and the animals were killed at intervals from one hour to 28 days after the injection. At autopsy, animals given doses in excess of 40 mg./kg. showed occasional lesions similar to those seen in the survivors from the previous experiment. Animals given smaller doses showed no apparent macroscopic abnormality.

On histological examination of the tissues from the latter group, diligent search was necessary to secure evidence of embolic phenomena, but it was occasionally found in the animals given the larger doses. All the animals, however, showed evidence of the deposition of metachromatic material in the reticulo-endothelial system. The maximum accumulation of material occurred between three and 24 hours after injection. The amount thereafter steadily diminished, but traces were still discernible at 28 days.

Interpretation of Histo-pathological Findings

The following tentative hypothesis was framed to collate these findings. Interaction of the large molecular weight dextran sulphates with fibrinogen occurs *in vivo* as *in vitro*, leading to the formation of an insoluble particulate precipitate in the blood-stream. Platelets and white-cells agglutinate and are enmeshed in the precipitate. If the process is of sufficient severity, arterioles and capillaries are embolized. When the material is administered intravenously, the main filter-bed is the pulmonary circulation. Abrupt blockage of a major portion of the pulmonary vascular tree produces sudden death attended by cardiac and respiratory embarrassment similar to that seen in human pulmonary embolism. More gradual occlusion of the pulmonary circulation, such as might be expected to occur with smaller doses, or doses absorbed more slowly via the peritoneum, leads to gradually increasing resistance to the output of the right ventricle. This explains the marked dilatation of the right side of the heart, the generalized cyanosis and the marked passive venous congestion of the abdominal organs in these animals.

The mechanism of production of the pulmonary oedema noted in many of these animals needs further elucidation. Pulmonary infarction caused by the blockage of arterioles is not sufficient to explain the intra-alveolar oedema found, not only adjacent to obvious infarcts, but also in areas in which no gross lesion was demonstrable. It is possible that capillary embolism may produce sufficient damage to the endothelial lining to cause increased permeability. The problem is being investigated further.

Doses, insufficient to produce immediate fatalities as outlined above, still produce embolism of sufficient severity to cause widespread infarction and haemorrhage. Whether or not the site of a particular micro-infarct serves also as the site of intractable haemorrhage appears to depend upon the physical characteristics of the tissues involved. Normally it may be supposed that tissue-damage, as the result of infarction, liberates large amounts of thromboplastin which initiate clotting of the effused blood. This, and the tissue-pressure of tightly-packed parenchymatous cells, together prevent the indefinite extension of haemorrhage. In animals given the large molecular weight dextran sulphates, the clotting-mechanism is arrested (due to thrombocytopenia, fibrinogenopenia and the strong antithrombin action of the compounds), but it is nevertheless notable that lesions in, for instance, the liver, remain localized, whereas those in loose mesenchymal tissues such as the mesen-

FIG. 8.—" Fibrin " embolism in rat spleen, following a dose of 200 mg./kg. of E size dextran sulphate. The splenic artery contains a laminated mass surrounded by small clumps of leucocytes. The mass stained positively with the " fibrin " stains and was also metachromatic to toluidine blue. Lendrum's acid picro-Mallory method. × 160.

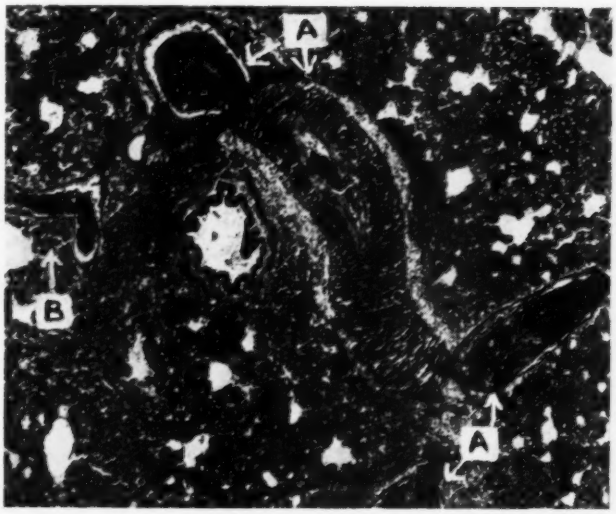


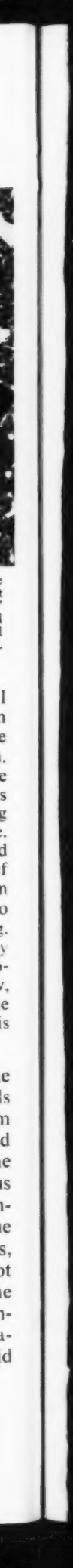
FIG. 9.—Rabbit lung following dose of 1,000 mg./kg. of E size dextran sulphate. A pulmonary artery and its branches, cut obliquely, show " fibrin " embolism. The branches marked " A " contain intensely fuchsinophil material. The branches marked " B " contain leucocytes apparently clumped to form the tail of the main embolus. Lendrum's acid picro-Mallory method. × 60.

tery, omentum and retroperitoneal tissues are characterized by massive haemorrhage.

Embolism of still less extent results in non-fatal infarction and haemorrhage. The production of minimal intravascular precipitation allows clearing of the circulation by the phagocytic activity of both fixed and free cells of the reticulo-endothelial system, and accounts for the apparently selective storage of the material in the reticulo-endothelial tissues of the liver, spleen and bone-marrow. Deposition of metachromatic material in the reticulo-endothelial cells would thus constitute the earliest indication of " toxicity " in the sense of indicating that higher dosage of a compound producing this effect might be expected to produce a bleeding tendency by the mechanism outlined.

It is necessary to point out that this interpretation is at variance with that of certain other authors. For instance, Asplund, Borell, and Holmgren (1939) reported the accumulation of metachromatic material in the reticulo-endothelial tissues of rats, rabbits, and guinea-pigs given large doses of heparin, and regarded this as the normal method of disposal of the substance. The heparin used was prepared from beef liver by the authors themselves. No details of the purification of the material nor of the chemical or physical characteristics of their final product were given. Judging from its activity, it would appear to have been a fairly crude preparation which may well have contained a substantial amount of chondroitin sulphate, which Jorpes and Gardell (1948) have shown to be a common contaminant of heparin. Piper (1945b) showed that chondroitin sulphate formed insoluble complexes with fibrinogen, and is stored in reticulo-endothelial cells in which a strong metachromatic reaction is consequently demonstrable. Personal attempts to reproduce the results of Asplund *et al.*, using commercial heparin (prepared from beef *lung*) obtained from five different sources, have been uniformly unsuccessful. Single injections of up to 3 g./kg. and thrice daily injections of 20 mg./kg. given to rats did not result in the deposition of any observable metachromatic material in the reticulo-endothelial cells of the liver, spleen or bone-marrow, though metachromatic granules were seen in the proximal convoluted tubular epithelium in this species.

Further investigation of the nature of the material deposited within reticulo-endothelial cells and upon the surface of the vascular endothelium provided support for the present hypothesis and information concerning the disposal of the material. When serial sections taken from various organs of animals given the D and E size compounds were stained alternately with toluidine blue and one or other of the various " fibrin " stains, it was found that the intravascular material not only gave a metachromatic reaction with toluidine blue, azure A or thionin, but was also Gram-positive, phloxinophilic with the phloxine-tartrazine method, fuchsinophilic with the acid



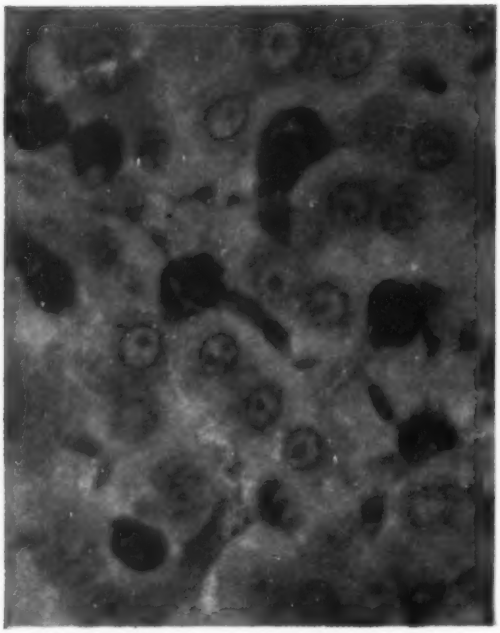


FIG. 10.—Liver, showing Kupffer cells loaded with granules of metachromatic material. Rat given 100 mg./kg. E size dextran sulphate. Toluidine blue. × 800. Kodak Ektachrome.

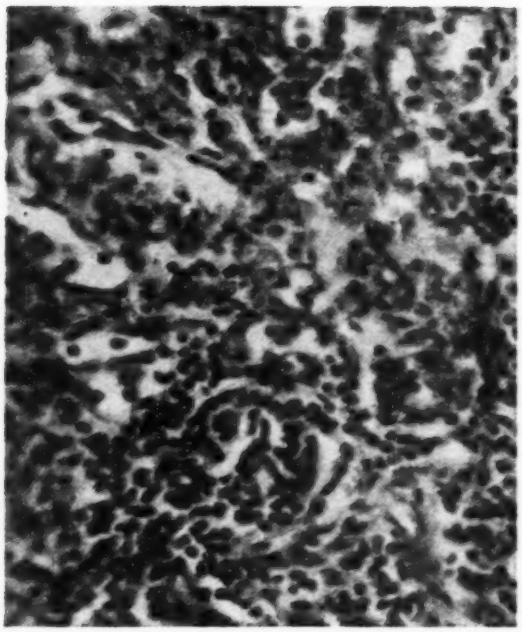


FIG. 11.—Spleen. Vacuolated phagocytes lining the pulp-sinuses contain metachromatic material. Rat given 100 mg./kg. E size dextran sulphate. Toluidine blue. × 700. Kodak Ektachrome.

FIG. 12.—Rabbit liver following dose of 1,000 mg./kg. of E size dextran sulphate. A large portal vein contains a "fibrin" embolus consisting of a condensed, coarsely fibrillar, intensely fuchsinophil portion continuous with a more delicate network of fuchsinophil material enmeshing leucocytes. Lendrum's acid picro-Mallory method. × 1770. Kodak Ektachrome.

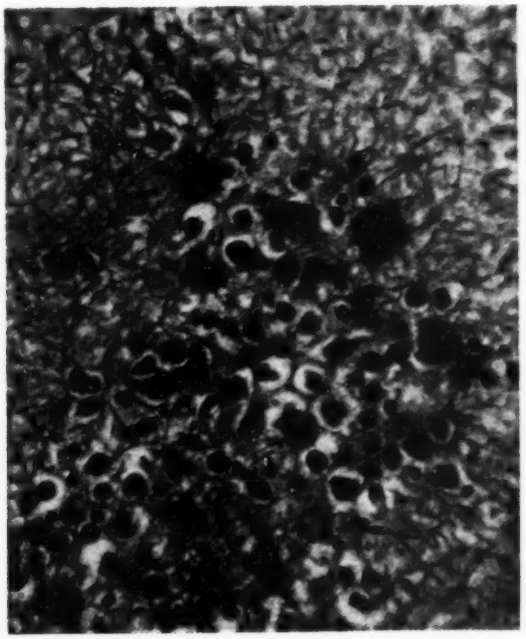


FIG. 13.—High power view of portion of section shown in Fig. 12, showing the nature of the open network enclosing leucocytes and platelets. Lendrum's acid picro-Mallory method. × 320.

[*Facing p. 9*

picro-Mallory method and stained dark blue with phosphotungstic acid haematoxylin—that is, gave the staining reactions of fibrin or fibrinogen (Figs. 8 and 9). Occasionally, in large vessels which were not completely occluded, the intravascular mass could be seen to consist of a coarsely fibrillar portion continuous with a more delicate fibrillary network containing, enmeshed within it, clumps of leucocytes and faintly-staining granular material presumed to be the debris of disintegrated platelets (Figs. 12 and 13). In animals surviving for a week or longer, the intravascular material was seen to become endothelialized and to undergo organization in a manner closely similar to that which occurs in a thrombus. In the later stages the appearance was very like that of an atheromatous deposit.

The granular material within the reticulo-endothelial cells also gave both a metachromatic reaction and a positive staining reaction for fibrin or fibrinogen, if the animal had been killed within ½–4 hours of injection. But in animals allowed to survive for 24 hours or longer the material no longer gave a positive staining reaction for the protein, although, as already indicated, a metachromatic reaction with toluidine blue persisted for 28 days or longer. An experiment kindly performed by Mr. Henry Finck, under the supervision of Professor Isadore Gersh, in the Department of Anatomy of the University of Chicago, indicated that this result was probably not due to diffusion of the protein component owing to the action of the solvents, etc., used during the fixation and embedding processes.

A rat was injected intraperitoneally with a dose of 10 mg./kg. of the D size dextran sulphate. Twenty-four hours later the rat was killed and portions of the liver and spleen prepared by freezing and drying. Paraffin sections were made. Material was visible in many tissue phagocytes, in some of them as granular accumulations. Examination under the ultra-violet microscope and measurement of the absorption at a wave-length of 2,800 Å gave no indication of the presence of protein in the phagocytozed material. According to Gersh (personal communication) this should not be taken to mean that no protein was present, but simply that not enough was present to be detectable by this procedure.

The reticulo-endothelial cells are known to contain active proteolytic enzymes and the dextran sulphate-fibrinogen complex has previously been shown to be easily dissociable, even *in vitro* (Walton, 1952). The above results were therefore interpreted as indicating that the protein moiety of the phagocytozed material was split off and digested quickly, leaving the charged polysaccharide component to undergo much slower catabolism. This is in keeping with the findings of Kaplan, Coons, and Deane (1950) and of Coons, Leduc, and Kaplan (1951), who showed that even heterologous and foreign proteins disappear considerably more quickly from reticulo-endothelial cells than similarly phagocytozed polysaccharides.

The material in the cells of the proximal convoluted tubules of the kidneys of rats treated with the D and E size dextran sulphates gave the staining reactions of the sulphated polysaccharide and of protein but not of fibrinogen. The significance of this observation will be discussed elsewhere.

Relation Between Molecular Weight and Toxicity

These histological findings were taken to confirm the theory that the apparently different toxic manifestations seen at different dosage levels all derived ultimately from the formation of insoluble complexes between these compounds and fibrinogen. From this it followed that one might expect lowered toxicity as the molecular weight of the compound decreased and the tendency to precipitate fibrinogen lessened. It will be seen that this has already been demonstrated, since it was shown that larger doses of the E size compound were necessary to produce changes equal in severity to those evoked by any given dose of the D size compound.

It also followed that " contamination " by large molecules of a sample of dextran sulphate of low *average* molecular weight might be expected to add appreciably to its toxicity. This has now been shown to be so (Ricketts and Walton, 1953). For this reason, the sample of the I size dextran sulphate used for the quantitative and qualitative comparisons described below was carefully refractionated to ensure the maximum possible homogeneity of molecular size.

The relation between molecular weight and toxicity was given rough numerical expression by assessment of the LD50's of the dextran sulphate in relation to heparin. The results derived from probit analyses of the survival of groups of mice during the seven days following intravenous injection are shown in Table I. 240 mice were used, in groups of 60 for each compound and of 10 for each level of dosage.

Grönwall *et al.* (1945) had previously demonstrated decrease of toxicity with decrease of the molecular weight of the dextran sulphates. Direct comparison with their results is not possible, since they expressed the toxicity of their compounds in terms of an undefined " lethal dose to rabbits." Nevertheless, in general terms, the present observations seem to agree with theirs.

TABLE I

COMPARISON OF THE TOXICITY OF INTRAVENOUS HEPARIN AND DEXTRAN SULPHATES IN MICE

Compound	Average Molecular Weight	LD50* (g./kg.)	Fiducial Limits of LD50	Slope of Curve ††	Standard Error of Slope
D size	458,000	0·154	0·19–0·12	5·1834	1·206
E size	47,000	0·573	0·64–0·52	8·3083	2·416
I size	7,500	2·120	2·72–1·66	3·6396	0·862
Heparin	? 17,000	2·030	2·70–1·53	3·7488	0·833

* Derived from probit log. dose relationship which was approximately linear.

† Comparison of the significance of the differences between slopes (at 5% level), taken in pairs, showed:—

I size/Heparin: $t = 0·1$; $p = 0·9$
D size/I size: $t = 1·04$; $p = 0·3–0·2$
D size/E size: $t = 1·16$; $p = 0·3$
E size/I size: $t = 1·82$; $p = 0·1–0·05$

The differences were therefore not regarded as statistically significant.

Although the LD50 of the I size dextran sulphate was roughly equal, weight for weight, to that of the heparin sample used, this was not taken to imply equal toxicity of the two compounds. The lower anticoagulant potency of this dextran sulphate necessitates the use of a larger absolute weight of material to obtain a quantitatively equal effect on the clotting-time. Its toxicity might therefore be regarded as correspondingly greater than that of heparin. But there are certain objections to accepting this method as a satisfactory means of measuring the toxicity of these compounds.

Although a quantal response test was employed here for comparative purposes, it is clear that this technique was not an informative guide to the true toxicity of the E and D size dextran sulphates. Even at dosage levels between one-twentieth and one-fiftieth of their respective LD50's, these compounds could be shown to set in train some or all of the pathological effects described above. Nor was the test satisfactory as a means of assessing the toxicity of the small molecular weight dextran sulphate relative to that of heparin. The deaths produced by doses of 2 g./kg. or more of heparin, or the I size dextran sulphate, all resulted from haemorrhage. No evidence was obtained that this was initiated by embolism or infarction (see below). Animals given doses in excess of 1 g./kg. of either compound developed subcutaneous and occasional intramuscular haematomata at points where they had been subjected to comparatively minor traumata. Subcutaneous, intramuscular, and intraperitoneal injections were often attended by the formation of haematomata around the needle track, and these were sometimes sufficient to produce exsanguination.

In some instances haemorrhage occurred in animals given very large doses of heparin or the I size dextran sulphate without any obvious externally applied injury. It is generally agreed that, under normal conditions, many breaches of continuity of the vascular endothelium occur as the result of "wear and tear," and that these are quickly sealed by the ordinary haemostatic mechanism. The interference with this mechanism by enormous doses of any anticoagulant may result in prolonged oozing from minute injuries. Jaques, Charles, and Best (1938) pointed out that in small laboratory animals, such as the rat and the mouse, such lesions are poorly tolerated since, in relation to the small total blood volume of these animals, a seemingly small blood-loss may be sufficient to produce profound haemodynamic effects. At very high levels of dosage of these compounds the mortality rate in mice is probably, therefore, an index of the incidence of chance encounters with trivial injury rather than a measure of the "toxicity," in the accepted sense, of the compounds.

Single intravenous doses in excess of 25 mg./kg. of heparin are rarely, if ever, administered to man, in whom the therapeutically effective dose is about 1–2 mg./kg. (Jorpes, 1946; Duff, Linman, and Birch, 1951). The effective dose of the I size dextran was found to be 6–8 mg./kg., so the main efforts were directed to assessing its toxicity at doses between this level and 200 mg./kg., on the assumption that this represented an ample therapeutic range.

Investigation of the Small Molecular Weight Dextran Sulphate

It has already been shown that this compound did not affect the stability of plasma fibrinogen *in vitro* and that no significant alteration of the levels of the formed elements of the blood or of the plasma fibrinogen occurred *in vivo* (see above). When doses between 5 mg./kg. and 200 mg./kg. were administered intravenously to mice and rats, no observable alteration of physiological function occurred, other than an increase in the clotting-time. Doses between 20 mg./kg. and 1,000 mg./kg. were equally well tolerated by rabbits. Doses between 20 mg./kg. and 80 mg./kg., given intravenously to an anaesthetized dog by Dr. J. A. Barclay, caused no alteration of blood-pressure, pulse-rate or respiratory rate. Doses between 40 mg./kg. and 80 mg./kg., similarly administered to two baboons by Dr. P. L. Krohn, produced no untoward effects. Histological examination of tissues from these animals did not show any storage of metachromatic material in reticulo-endothelial tissues.

Examination of sections taken from the liver, spleen, kidneys, skeletal and cardiac muscle, skin, bone-marrow, lymph-nodes, thyroid, adrenals, pancreas, gonads and brain of mice, rats and rabbits treated with the small molecular dextran sulphate showed no evidence of parenchymal damage, though this was looked for after single injections, repeated injections and over periods

TABLE II

EFFECT OF FOUR DAILY INTRAVENOUS INJECTIONS OF 10 MG./KG. OF I SIZE DEXTRAN SULPHATE IN RABBITS

	Platelets per c.mm.	Leucocytes per c.mm.	Haemoglobin %	Fibrinogen g./100 ml.	Urine		Coagulation-time*	
					Albumen	Deposit	Maximal	Minimal
Before injections	320,000	9,800	110	0·34	Nil	Nil	3 min. 10 sec.	2 min. 5 sec.
1st day ..	362,000	10,600	110	0·36	Nil	Nil	75 min.	3 min. 50 sec.
3rd day ..	334,000	8,400	109	0·32	Nil	Nil	90 min.	4 min. 15 sec.
5th day ..	376,000	10,200	112	0·35	Nil	Nil	82 min.	4 min.

* Coagulation-times performed by Dale and Laidlaw's method at 37° C.
" Maximal " clotting-times taken 5 minutes after injection.
" Minimal " clotting-times taken immediately before next injection.

from one hour to six months after the last injection. These results are illustrated by the following typical experiment, designed to simulate a short therapeutic course of injections:

Three rabbits were given four-hourly injections during the daylight hours (four injections daily) of 10 mg./kg. for five days. Before the injections were started the following base-line observations were made—estimation of fibrinogen and total plasma proteins, platelet and white-cell counts, haemoglobin estimation, clotting-time, examination of urine for albumen, and of the centrifuged deposit microscopically for red-cells, casts, etc. These estimations were repeated at intervals throughout the experiment with the results shown in Table II. During the course of the experiment the animals showed no untoward reaction to the injections, and at its conclusion they were alive and well. One animal was killed then, and the remainder were killed at three-week intervals subsequently. Post-mortem examinations showed no macroscopic abnormalities. Examination of sections taken from the liver, spleen, lungs, heart, kidneys, endocrines, and brain showed no histological abnormalities.

Attempts to sensitize guinea-pigs to the I size dextran sulphate were unsuccessful:

Twelve adult male guinea-pigs weighing between 450 g. and 600 g. were injected daily with 0.5 ml. of a 2% solution of an I size dextran sulphate, intraperitoneally, for six days. A fortnight was allowed to elapse and then each animal was given 0.2 ml. of the same solution intravenously. None of the animals showed any evidence of an anaphylactic reaction. Intracutaneous injection of the same material did not evoke whealing.

On the other hand, the parenteral administration of the D and E size compounds or of unusually polydisperse samples of low average molecular weight to guinea-pigs was attended by a transitory anaphylactoid reaction. The further investigation of this phenomenon and its possible practical significance has been reported elsewhere (Walton and Ricketts, 1954).

DISCUSSION

The toxicity of chondroitin sulphate and of synthetic sulphuric esters of chitin, cellulose, starch, xylan, and alginic acid has been investigated by Astrup, Piper, and their colleagues (Astrup, Galsmar, and Volkert, 1944; Astrup and Galsmar, 1944; Astrup and Piper, 1945, 1946; Piper, 1945a, 1945b, 1946a, 1946b, 1947; Barsøe and Selso, 1946; and Astrup, 1953) with results in substantial agreement with the present findings. All these compounds were found to agglutinate the platelets and some of them, in high dosage, were reported to produce embolic phenomena and haemorrhages in experimental animals. The detailed mechanism of production of such changes is suggested by the present work on dextran sulphates of varying molecular size. In agreement with Astrup (1953) the toxicity of polysaccharide sulphates is held to be dependent ultimately upon the aggregation of blood-cells which some of them produce. With the dextran sulphates, this process has been shown to be dependent upon insoluble complex formation with the plasma proteins, since none of the dextran sulphates produced any effect upon platelets or other suspended particles in the absence of protein from the suspending medium, and since homogeneous samples of small molecular weight, which failed to precipitate fibrinogen, produced no effect upon the blood cells even in whole plasma (Walton, 1953).

In vivo, the large molecular weight dextran sulphates have been shown to cause thrombocytopenia, leucopenia, succeeded by leucocytosis and fibrinogenopenia. With increasing dosage, the formation of intravascular deposits, consisting of agglutinated platelets, leucocytes and fibrin has been demonstrated. Since these deposits occurred in spite of virtually complete arrest of the normal clotting mechanism, they were assumed to arise from the same process of non-specific complex formation with the plasma proteins which had previously been reported to occur *in vitro* (Walton, 1952, 1953). This formation of apparent thrombi or emboli by a mechanism independent of the ordinary clotting-process is an unfamiliar concept in pathology and is apparently paradoxical behaviour for material showing anticoagulant acti-

vity. Whether this has any relevance to the general problem of thrombosis and embolism is conjectural. There is, however, a considerable body of evidence to indicate that such behaviour is not confined to sulphated polysaccharides but occurs even with uncharged polysaccharides and other macromolecules.

It has been shown by Ricketts (1952b) that neutral dextrans, if of sufficiently large molecular size, form insoluble complexes *in vitro* with fibrinogen in plasma and in pure solution. Fletcher, Martin, and Ratcliffe (1952) have shown that polyvinylpyrrolidone, acacia and laminarin also interact with fibrinogen in a similar manner.

Staub, Mezy, and Golondas (1938) described the occurrence of leucopenia and agglutination of leucocytes in rabbits given glycogen and gum acacia. This was confirmed and extended by Bucher (1939), who observed that platelets were also affected. He noted that rabbit liver glycogen was less effective in producing these changes than glycogen derived from pig's liver, speculating on whether these differences could be correlated with the known differences in molecular weight of the preparations.

A thorough investigation of the pathogenicity of macromolecular substances was carried out by Hueper (1942, 1943). He found that gum arabic and gum acacia, pectin, methyl cellulose and polyvinyl alcohol, glycogen, gelatin and ovalbumin all produced what he called the " macromolecular haematic syndrome." This comprised (1) primary transitory leucopenia, (2) secondary myeloid leucocytosis, (3) simple anaemia, (4) an accelerated erythrocyte sedimentation rate, and (5) in some instances, an increased coagulation time. Intravascular deposition of these materials was demonstrable, in many cases, as emboli occluding pulmonary, renal and other vessels in the acute lesions and as endothelialized plaques, resembling atheromatous deposits, in the chronic ones. The non-proteinous compounds were demonstrable in reticulo-endothelial cells in the liver, spleen and bone-marrow. The close resemblance between these and the present findings needs no stressing.

Miscellaneous macromolecular colloids have been used to provoke the so-called " generalized Schwartzman reaction " by Thomas and Good (1952), Good and Thomas (1952), and Thomas, Denny, and Floyd (1953). These materials included glycogen, acacia and bacterial polysaccharides, and they produced clumping of the platelets and leucocytes with the arrest of the clumps in the capillary beds of internal organs. The process was attended by acute circulatory collapse with widespread embolism and infarction. The eosinophilic material producing the lesions gave the staining reaction of " fibrinoid " and was thought to be mucopolysaccharide in nature. From these authors' published descriptions and illustrations, the lesions of the generalized Schwartzman reaction appear to resemble closely those obtained with the large molecular weight dextran sulphates.

This " family resemblance " between the lesions produced by widely differing macromolecular materials leads one to conclude that the toxic manifestations produced by some of them may be closely related, in mechanism of production, to that suggested for the large molecular weight dextran sulphates. These last, and other toxic polysaccharide sulphates, probably only differ from other macromolecular materials in that their additional antithrombin action also arrests the normal clotting mechanism, thereby adding the further hazard of haemorrhage to an organism already imperilled by embolism and infarction.

The present finding, that reduction of the molecular size of a sulphated polysaccharide reduces or abolishes the train of pathological events produced by its analogues of greater molecular weight, is probably of importance in the design of heparin-substitutes. It should be stressed that although it has proved possible to produce a substitute for heparin of low toxicity and qualitatively similar clinical efficacy (Ricketts, Walton, *et al.*, 1953), it is not claimed that this is the only or the best starting material which can be employed. The physical characteristics distinguishing a dextran sulphate of low toxicity are probably not transferable directly to other materials. However, since the few crude unfractioned polysaccharide sulphates (prepared from other starting materials) which have been personally examined have shown undesirable properties similar to those of the large molecular weight dextran sulphates, it seems necessary to urge again the advisability of biological screening and of definition of the physical characteristics of other compounds of this kind proposed for therapeutic use.

Summary

1. The toxicity of sulphuric esters prepared from three dextrans of widely differing molecular weight is compared with that of commercial heparin.

2. The dextrans of large molecular weight—average above 40,000—are more toxic than heparin. The toxicity increases with increase of molecular weight.

3. The toxicity is considered to depend on the effects of these compounds upon the plasma-proteins and the formed elements of the blood.

4. At low dosages, the large molecular weight dextran sulphates form insoluble complexes with fibrinogen. The particles of this complex are removed from the circulation by the phagocytic activity of reticulo-endothelial cells without evidence of serious harm to the organism in acute experiments.

5. At higher dosages, agglutinates of the formed elements of the blood are demonstrable as conglutination thrombi or emboli in blood-vessels. As a result, infarcts of micro- or macroscopic dimensions can be demonstrated in various organs. Simultaneous interference with blood-clotting by the antithrombin action of these compounds possibly lessens, but does not prevent, such changes, and sometimes leads to intractable haemorrhage from the sites of infarction. Still greater dosage produces a rapidly fatal termination from embolism itself.

6. Attention is drawn to the behaviour of other macromolecular materials which have been reported to produce a sequence of events similar to that described above, suggesting a common mechanism of action.

7. On the other hand, the small molecular weight dextran sulphates—compounds with an average molecular weight of about 7,500—show qualitatively similar anticoagulant action to that of heparin and comparable low toxicity.

8. These compounds do not affect the plasma proteins or the formed elements of the blood and produce no evidence of embolism in experimental animals. As with heparin, massive doses are required to produce death from spontaneous bleeding. They therefore appear likely to be equally safe for therapeutic use as anticoagulants.

It is a pleasure once more to acknowledge the advice, assistance, and collaboration of my colleague, Dr. C. R. Ricketts, in various aspects of this investigation, and to thank Professor J. R. Squire for his encouragement, his helpful criticisms as the work progressed, and for his assistance with the manuscript. I am grateful to Professor G. R. Cameron, F.R.S., for his opinions upon some of the pathological problems which arose and for his general interest in this project. Thanks are due to Drs. J. A. Barclay and P. L. Krohn for kindly consenting to use dextran sulphate in the course of their own experimental work on dogs and baboons respectively. I am indebted to Professor I. Gersh and Mr. H. Finck for preparing and examining freeze-dried preparations of tissues from animals given dextran sulphate. A sample of Heparin "Leo" was kindly supplied by Mr. E. G. Tomich, of Glaxo Laboratories, Ltd., a sample of Heparin (Lederle) by Professor M. Stacey, F.R.S., and a sample of "Paritol" by Mr. T. B. Wallace, of Smith, Kline, and French, Inc. The dextran from which the preparations examined were made was a gift from Dextran, Ltd. The photomicrographs were the work of Mr. F. Bradley. I am grateful to Miss A. Fisher for the probit analyses and statistical calculation of the LD50's of the various compounds.

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INTERACTION OF NICOTINE AND ESERINE, EPHEDRINE, ATROPINE, HEXAMETHONIUM, AND ADRENALINE IN ISOLATED GUINEA-PIG AURICLES

BY

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The mixed effect (inhibition and stimulation) of nicotine on isolated hearts is well known. That nicotine acted on nervous tissue was shown by the antagonistic effect of apocodeine (Dixon, 1920), and confirmed by experiments on isolated rabbit auricles (Gronchi, 1939). During experiments planned to study the action of various drugs on the mechanical and electrical behaviour of isolated guinea-pig auricles, we observed that hexamethonium completely antagonized nicotine; this was a further confirmation of Dixon's interpretation. There were, however, some minor points which seemed to deserve further investigation, such as the specificity of the action of hexamethonium on nervous tissues in the concentrations which antagonize nicotine; whether chemically different ganglionic blocking agents also antagonize nicotine; the effect on the action of nicotine of pretreatment with anticholinesterase drugs (which should potentiate the inhibitory action) and with ephedrine (which should antagonize the stimulant action); and the mechanism of the progressive change of auricular response to nicotine in relation to the duration of survival of the preparation. During these studies nicotine revealed itself as an excellent compound for investigating whether the autonomic nervous system participates, as was presumed, in the progressive exhaustion of the auricles, and whether there exist compounds able to restore its excitability. Adrenaline proved very effective in restoring the inhibitory action of nicotine.

In the meantime a paper was published on the stimulation of isolated rabbit auricles by substances which stimulate ganglia (Kottegoda, 1953), in which the antagonistic action of hexamethonium to nicotine stimulation was clearly illustrated and its mechanism discussed. A comparison of Kottegoda's results and mine showed that rabbit and guinea-pig auricles react very similarly to nicotine and hexamethonium. The reader is therefore referred to Kottegoda's paper for qualitative information about the hexamethonium - nicotine antagonism.

METHODS

Auricles were isolated from guinea-pigs weighing 300–400 g. and suspended horizontally in a bath containing well-oxygenated Ringer solution, at 29° C. (Giotti, 1953). The contractions were recorded isotonically under slight tension. Although the auricles contract vigorously immediately after immersion in the bath, experiments were not usually started until one hour later. The measurement of drug effects required that the preparation should be in a steady state; this was maintained, despite frequent washings, for many hours. Guinea-pig auricles were selected, instead of the more commonly used rabbit auricles, on the assumption that the smaller wall thickness would allow better diffusion of drugs and metabolites. Diffusion is better (Schmid, Siess, and Bühler, 1952) if auricles of young guinea-pigs are used. No differences were observed between the actions of freshly prepared solutions of "Nicotinum purissimum Merck" neutralized with 0.01 N HCl and dissolved in the buffered perfusion fluid, of the same solution purified according to Forst's procedure (1943), and of freshly prepared solutions of nicotine acid tartrate whether treated or not treated with charcoal. This agrees with the observations of Larson, Finnegan, Van Slyke, and Haag (1950). Doses are expressed as the final concentration in g./ml. in the bath fluid.

RESULTS

Action of Nicotine on Untreated Fresh Auricles.—Nicotine 2×10^{-8} has no effect on the rate and increases slightly, but inconstantly, the amplitude of contraction, as observed over a period up to 20 min. Effective concentrations lie between 2×10^{-7} and 2×10^{-6}. The action appears with a short latency (8–10 sec.); reduction of rate and amplitude predominate at first, but later there is usually an increase of amplitude. Nicotine 2×10^{-6} admin-

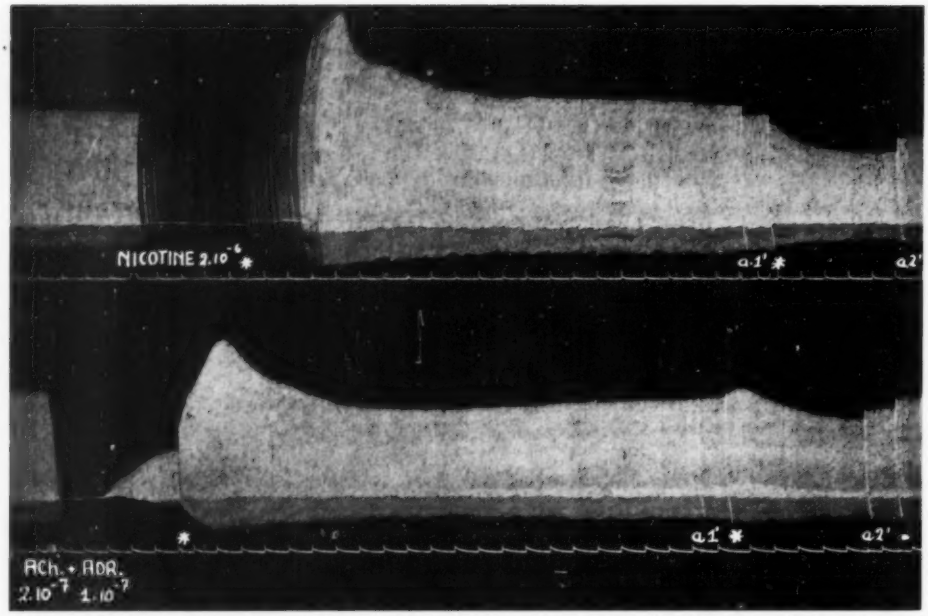

FIG. 1.—Isolated guinea-pig auricles. Similarity in the effects of nicotine (upper tracing) and acetylcholine + adrenaline (lower tracing). Washings are indicated by *. Time, 20 sec. a.1, recording stopped for 1 min.

istered repeatedly gives reproducible results if the administrations are interrupted by washings, and exposure is not prolonged beyond 10 min. If the auricles are left in contact with higher concentrations, and for longer periods, the response may become progressively smaller. The effect of washing differs according to the phase of nicotine action. During inhibition, washing produces a large increase of rate and amplitude; during stimulation it causes a decrease of amplitude, and an increase, or no change, of rate. The course and quality of the action of nicotine, and of subsequent washing, may be almost exactly duplicated by the administration of an appropriate combination of ACh and adrenaline (Fig. 1).

Action of Nicotine on Fresh Auricles After Treatment with Other Drugs.—The unmasking or potentiation of the stimulant action of nicotine by atropine is well known. Fig. 2 shows the potentiation by eserine of the decrease of rate and amplitude caused by nicotine. This potentiation and its antagonism by atropine point to an effect mediated through ACh.

The possibility that the stimulant effect of a drug on an isolated heart may be mediated through the liberation of sympathin-like substances has been substantiated by much experimental evidence. Fig. 3 shows that ephedrine antagonizes the stimulant effect of nicotine after atropine, just as it antagonizes the similar effect of adrenaline. Competition between ephedrine and sympathin for common receptors seems an adequate explanation.

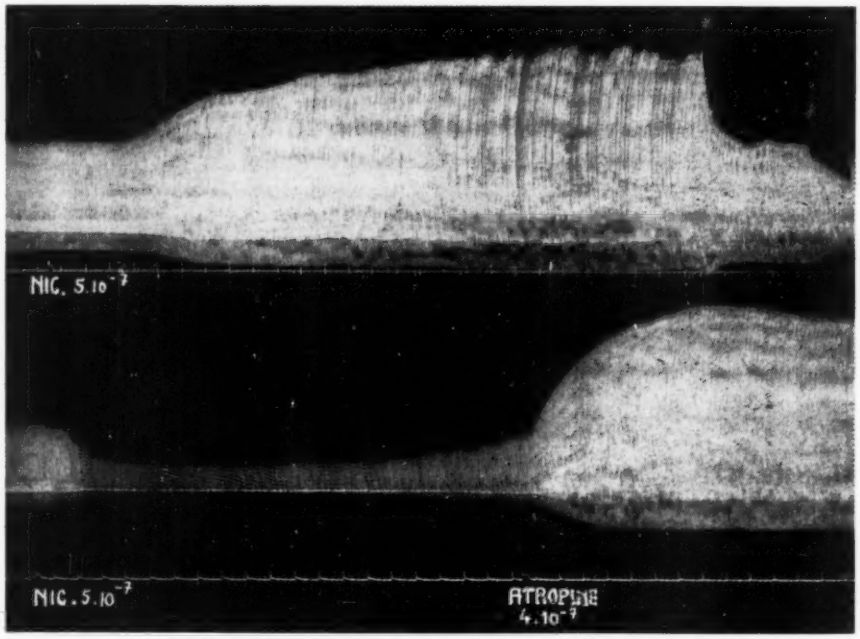

FIG. 2.—Isolated guinea-pig auricles. Interaction between nicotine, eserine, and atropine. The upper record shows the action of nicotine on untreated auricles; the lower record shows that nicotine after eserinization (eserine sulphate 2×10^{-8} in the bath) has an inhibitory action which is reversed by atropine. Interval between eserine and nicotine administration was 45 min. Time, 20 sec. * indicates washing.

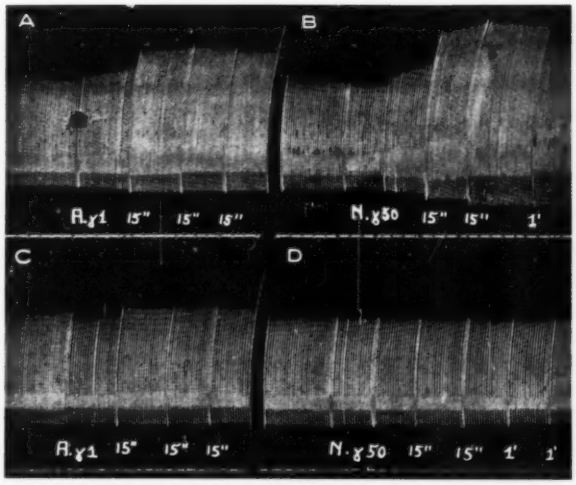

FIG. 3.—Isolated guinea-pig auricles in atropine sulphate 2×10^{-7}. Antagonistic action of ephedrine towards nicotine. A shows the effect of adrenaline 1 μg.; B that of nicotine 50 μg. In C and D adrenaline and nicotine were repeated after ephedrine hydrochloride 2.5 mg. Washings at the end of A, B, and C. Time, 2 sec.

A direct action of nicotine on muscular effectors (Gronchi, 1939), or at a postganglionic level (Tripod, 1949), has also been claimed to explain peculiarities of its action. Kottegoda (1953) reported that hexamethonium (1.25×10^{-4}) inhibited the stimulation of rabbit auricles by nicotine. According to Kottegoda, hexamethonium does not modify the auricular response to adrenaline. In our conditions, hexamethonium 2×10^{-5} fully antagonized the stimulating action of nicotine 2×10^{-6} (after atropine 2×10^{-7}) without changing the response to injected adrenaline ($1–2 \times 10^{-7}$) or to $CaCl_2$ (twofold increase of the normal content of the bath fluid). Hexamethonium 2×10^{-5} also antagonized the inhibitory phase of nicotine action ($2 \times 10^{-7}–2 \times 10^{-6}$) which is evident in fresh untreated preparations. This concentration of hexamethonium did not modify either the response of the auricles to injected ACh (2×10^{-8}) or the negative inotropic effect of KCl (twofold increase of the normal content of the bath fluid), although it decreased the negative chronotropic action of KCl. Hexamethonium, in the range of concentrations ($0.2–2 \times 10^{-5}$) which partially or fully antagonized nicotine, had no direct action on either the mechanogram or the electrogram of the auricles. The partial antagonism between hexamethonium and KCl may be attributed to stimulation of ganglia by KCl (Feldberg and Vartiainen, 1935).

Atropine is known to have ganglionic blocking properties (Feldberg and Vartiainen, 1935; Marrazzi, 1939; Konzett and Rothlin, 1949, and many others). Atropine sulphate 1×10^{-5} completely prevents the action of nicotine 1×10^{-6}. A typical experiment is shown in Fig. 4; it is representative of many others performed with different auricles at various times after completing the preparation. This concentration of atropine is larger than that (2×10^{-7}) necessary to block the muscarine-like effect of a dose of ACh (2×10^{-8}) the action of which on rate and amplitude is similar to that produced by nicotine. The hexamethonium-like activity of atropine is graded according to the dose; 4×10^{-6} does not always completely prevent the action of nicotine 1×10^{-6}; when prevention is

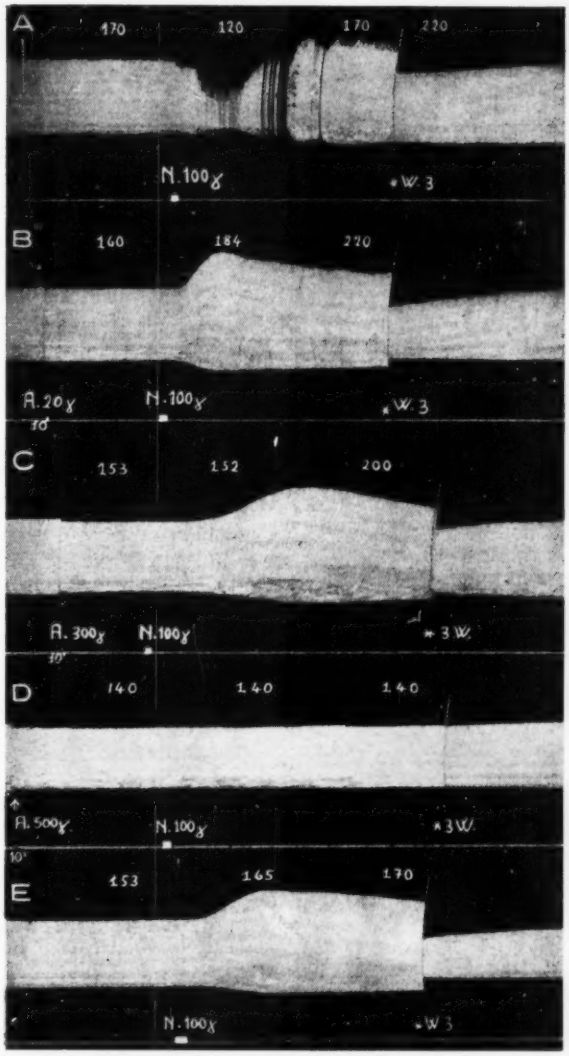

FIG. 4.—Isolated guinea-pig auricles. Influence of different doses of atropine on nicotine action. N, nicotine (as base); A, atropine sulphate; W.3, 3 washings. Numbers on the upper part of each record indicate auricular frequency determined from electrograms. Record A shows effect of nicotine (N), 100 μg. (as base); records B, C, and D show effects of the same dose of N after atropine sulphate (A) 20 μg., 300 μg., and 500 μg. respectively. Record E shows that the auricles still respond to nicotine despite the absence of effect after 500 μg. atropine.

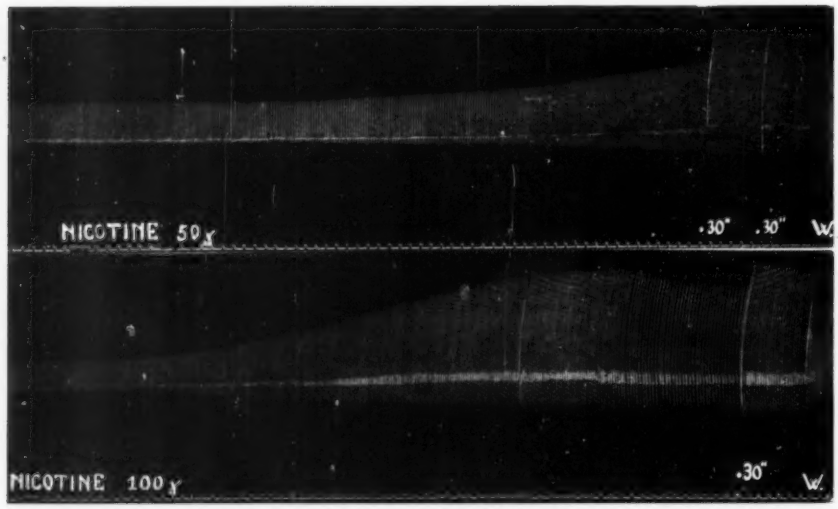

FIG. 5.—Hypodynamic isolated guinea-pig auricles (10 hours of work, several washings). Action of nicotine acid tartrate. W, washings; time, 2 sec. The upper tracing shows the effect of 50 μg. nicotine (as base) and the lower that of 100 μg. nicotine.

not complete there is a delay in the onset of nicotine action; protection is more pronounced towards the chronotropic than the inotropic action (see records C and E of Fig. 4). The action of atropine in antagonizing the chronotropic action of nicotine is also less reversible by washing than is that on the inotropic action. An interaction between atropine, and the sympathin liberated by nicotine, does not seem to play a role in these experiments: the inotropic and chronotropic responses of the auricles to adrenaline are not significantly changed after concentrations of atropine which act like hexamethonium.

Spadolini and Giachetti (1953) claim that the site of action of atropine, perfused in high concentration (1×10^{-5}) through Langendorff hearts, is upon excitatory ACh receptors, which they suppose to be present on myocardial effector cells and to be sensitive to very low concentrations of ACh (see also Spadolini and Domini, 1940). However, in our experiments, ACh added to the bath never stimulated guinea-pig auricles. ACh action was purposely investigated on 10 different preparations in concentrations from 1×10^{-15} to 1×10^{-7}; concentrations between 1×10^{-15} and 1×10^{-10} were ineffective; 1×10^{-9} and larger concentrations always gave the familiar muscarine-like effects. Eserinization did not alter the action qualitatively. Our results agree with Webb's (1950) on isolated rabbit auricles. The fact that nicotine stimulation is also prevented by ephedrine and hexamethonium is a further indication that its mechanism of action differs from that suggested by Spadolini and Domini for ACh. The results of experiments with ACh show furthermore that nicotine stimulation is not mediated through an effect of liberated ACh on the synthesis of ACh, such as happens when ACh causes stimulation of exhausted auricles (Bülbring and Burn, 1949; Burn, 1950).

Action of Nicotine on Hypodynamic Auricles.—In auricles exhausted by frequent washings and many hours of work the inhibitory actions of nicotine (1×10^{-6}), which may be predominant in fresh auricles, are less pronounced, or are replaced by a stimulant action (Fig. 5). This fact contrasts with the unchanged response to the minimal effective concentrations of ACh (1×10^{-9}–1×10^{-8}). Hexamethonium fully antagonizes stimulation by nicotine, as Kottegoda (1953) demonstrated in hypo-

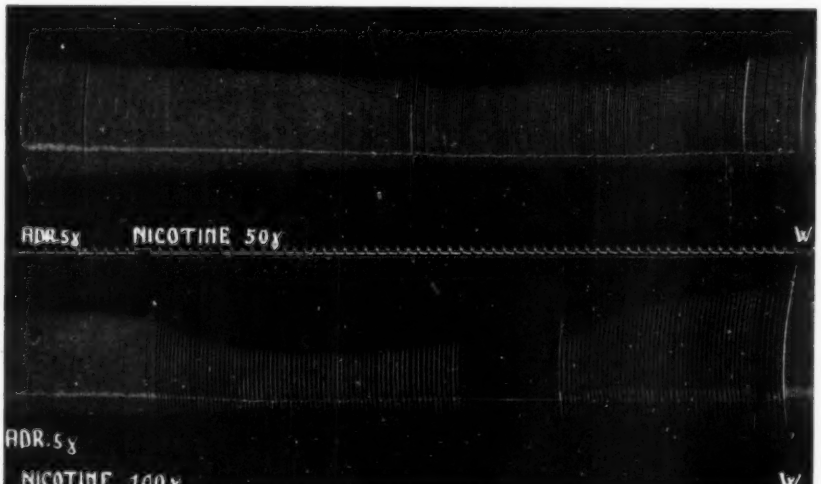

FIG. 6.—Hypodynamic isolated guinea-pig auricles (continuation of experiment of Fig. 5). The action of nicotine after adrenaline (ADR). The upper tracing shows the effect of 50 μg. nicotine after 5 μg. adrenaline, and the lower the effect of 100 μg. nicotine after 5 μg. adrenaline. Cf. with Fig. 5.

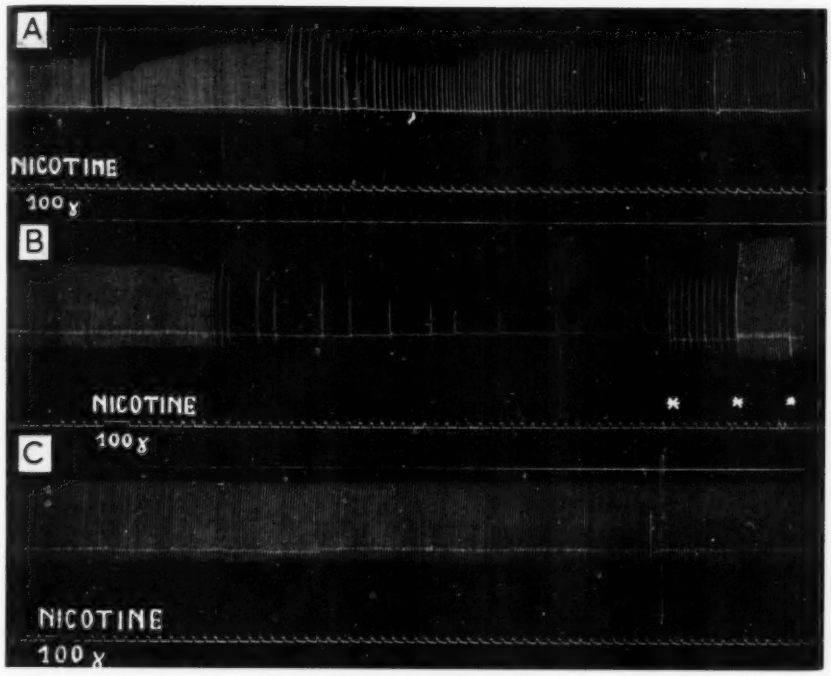

FIG. 7.—The action of nicotine on isolated guinea-pig auricles (A) before and (B) after treatment with adrenaline and (C) after treatment with adrenaline plus ephedrine. 5 μg. adrenaline given between A and B; between B and C 2.5 μg. ephedrine HCl and 5 μg. adrenaline. Adrenaline potentiates the inhibitory action of nicotine, but adrenaline plus ephedrine abolishes the action of 100 μg. N. Nicotine in C was added to the bath 5 min. after ephedrine and 30 sec. after adrenaline. In B, * denotes recording stopped for 1 min. At the end of every record 3 washings. Time, 2 sec.

dynamic rabbit auricles. After adrenaline, 1×10^{-7}, the response to nicotine is changed to a purely inhibitory one (Fig. 6; see, for a similar observation on rabbit auricles, Holz, 1938). If, however, the auricles are pretreated with ephedrine 1×10^{-5} the action of nicotine is prevented (Fig. 7). Hexamethonium is not so effective in protecting against the inhibitory action of nicotine in the presence of adrenaline as it is in protecting against the stimulant action in the absence of adrenaline (Figs. 8a and 8b): a concentration of 2×10^{-5} hexamethonium is necessary to prevent inhibition almost completely (Fig. 8a, line C), whereas 2×10^{-7} fully antagonizes stimulation (Fig. 8b). Hexamethonium 2×10^{-5}, however, does not prevent the inhibitory action of ACh in auricles which have been treated with adrenaline (Fig. 9). Atropine 2×10^{-7} fully prevents the inhibitory action of nicotine in the presence of adrenaline (Fig. 8a, line D).

DISCUSSION

The complex action of nicotine may be considered as the resultant of inhibition and excitation —in agreement with Dixon, 1920; Gronchi, 1939; Kottegoda, 1953, and others—with the possibility that one or other phase may predominate in different experimental conditions. Simultaneous stimulation of parasympathetic and sympathetic structures may explain this type of action, and also the effects of interaction with atropine, ephedrine, eserine, and hexamethonium. If nicotine stimulates autonomic ganglia without liberating ACh at the ganglionic level (Feldberg and Vartiainen, 1935), it is obvious that the antimuscarinic properties of atropine, and the anticholinesterase activity of eserine, may interfere with the action of the ACh liberated at post-ganglionic parasympathetic nerve endings. The stimulant action of nicotine on the sympathetic system has also been abundantly confirmed since the classical studies of Langley and Dickinson (for review, see Heubner, 1947). Evidence that the stimulant effect of a drug on an isolated heart preparation may be due to liberation of sympathin was provided for ACh (in the presence of atropine) by Hoffmann, Hoffmann, Middleton, and Talesnik (1945), and by McNamara, Krop, and McKay (1948). This view can be supported on anatomical, physiological and biochemical grounds. Thus there is chromaffin tissue in the heart (Trinci, 1907; Busacchi, 1912); the normal beating heart produces small quantities of sympathin (Külz, 1928); minced hearts treated with ACh produce a substance which inhibits atropinized rabbit intestine (McDowall, 1946); and heart extracts contain noradrenaline, adrenaline and hydroxytyramine (Goodall, 1951; Holtz, Kroneberg and Schümann, 1951). The antagonism by ephedrine of the stimulant effect of nicotine on atropinized auricles indicates that sympathin is implicated in the action of nicotine (De Jongh, 1951). The similarity between the effects of adrenaline and nicotine in atropinized auricles is evident in both mechanical and electrophysiological records. The following observations also agree with the concept that nicotine simultaneously stimulates both parts of the autonomic nervous system. Firstly, the biphasic type of action—inhibition followed by stimulation—is similar to that

seen on simultaneous stimulation of the vagus and accelerator nerves (Nelemans, 1951); secondly, the action of nicotine is very similar to the action of a mixture of ACh and adrenaline, and the washing-out effects are the same. Lawrentjew's (1929) and Woollard's (1926) opinion, that all intracardiac ganglia are parasympathetic, complicates the question where nicotine acts in the heart (for the nicotinic action of ACh on amphibian hearts, see Pick, 1920; Barlow, 1928; on isolated mammalian hearts, Hoffmann *et al.*, 1945; McNamara *et al.*, 1948). The problem of the site of nicotine action

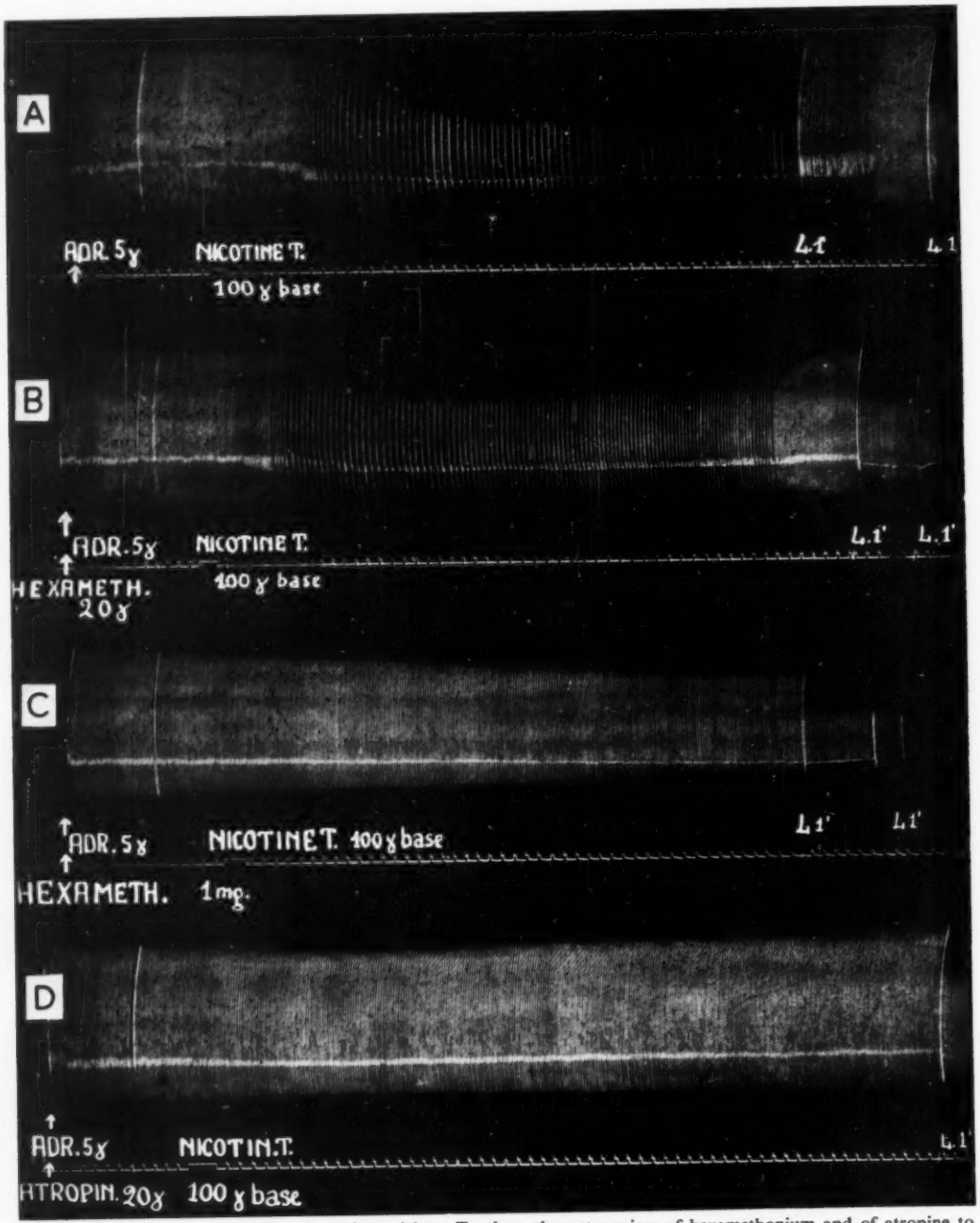

FIG. 8a.—Hypodynamic isolated guinea-pig auricles. To show the antagonism of hexamethonium and of atropine to the inhibitory action of nicotine after adrenaline. Time, 2 sec.; L 1', washings and stop of recording for 1 min.; ADR, adrenaline. Record A shows the effect of 100 μg. nicotine after 5 μg. adrenaline: records B and C show the effect of the same dose of nicotine after adrenaline plus 20 μg. and 1,000 μg. hexamethonium respectively. Record D shows the abolition of the nicotine effect. (Between B and C and C and D the response to 5 μg. adrenaline followed by 100 μg. nicotine remained as in A.)

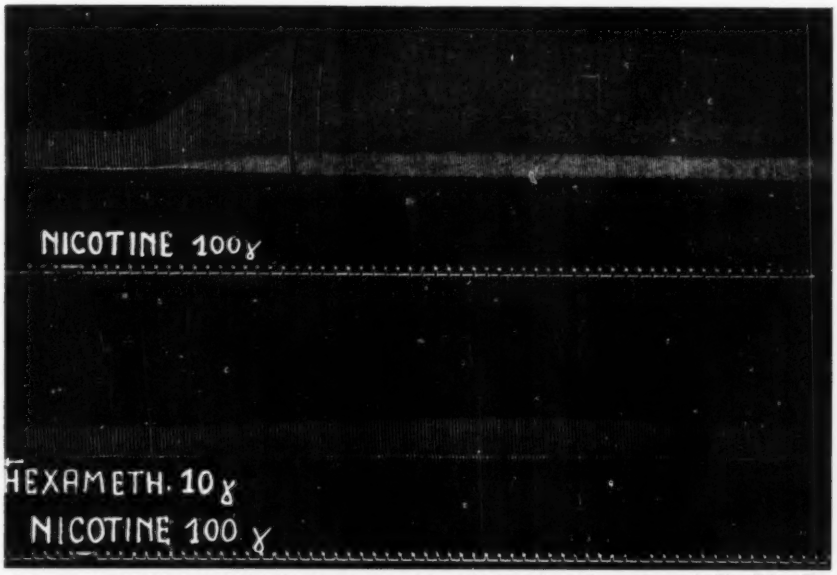

FIG. 8b.—Hypodynamic isolated guinea-pig auricles (continuation of experiment in Fig. 8a). To show the antagonism of hexamethonium towards the stimulating action of nicotine. Time, 2 sec.

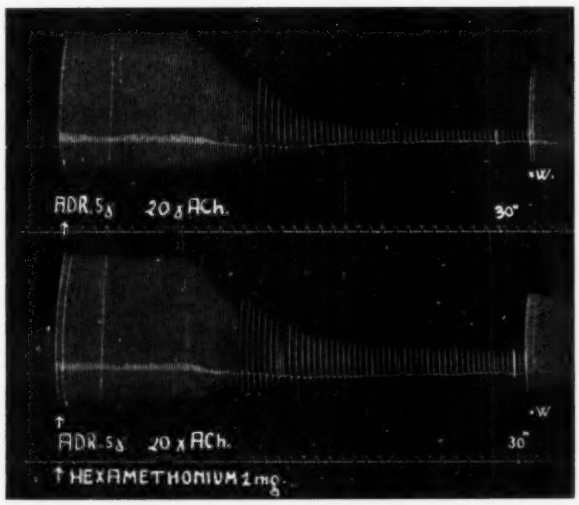

FIG. 9.—Hypodynamic isolated guinea-pig auricles. To show that hexamethonium does not antagonize the inhibitory action of acetylcholine (ACh) after adrenaline. Time, 2 sec.

applies to other tissues. Thus in the isolated ileum nicotine causes parasympathetic (motor) and sympathetic (inhibitor) effects ; inhibition of the ileum is evident when the nicotine is given after atropine or botulinum toxin (Ambache, 1951 ; Ambache and Edwards, 1951). Ambache and Edwards suggest the presence in the intestinal walls of adrenergic ganglion cells despite the lack of direct evidence. Applying the same hypothesis to my results, diagrams like those drawn by Dixon (1920) for the whole heart, and by Ambache and Edwards (1951) for the intestine, will also depict the site of action of nicotine on the auricles: inhibition is prevented by atropine and potentiated by eserine ; stimulation is unmasked by atropine and prevented by ephedrine ; both stimulation and inhibition are prevented by hexamethonium and by large doses of atropine. It is, however, questionable if full antagonism with hexamethonium proves the existence of a synapse in the preparation. If the action of hexamethonium is not restricted to the ganglion " but is fundamentally to prevent such excitation of nervous structures wherever it can be achieved " (Paton and Zaimis, 1952) there is no way to decide where exactly, in the heart, nicotine stimulation takes place (see also Kottegoda, 1953).

If the inhibitory phase of nicotine action in fresh untreated auricles is due, as seems likely, to the stimulation of parasympathetic ganglia, the progressive reduction in this inhibitory response during exhaustion may be explained by a decreased excitability of the parasympathetic ganglia or by a decreased sensitivity of effectors to liberated ACh. A decreased sensitivity to ACh does not seem of prime importance, because the response of auricles to injected ACh is not significantly reduced when they are so exhausted that nicotine stimulates them. Adrenaline may directly or indirectly restore the excitability of parasympathetic ganglia, and consequently the inhibitory response to nicotine, to a level similar to or greater than that of fresh preparations. This hypothesis agrees with the well-known action of small doses of adrenaline on the excitability of the cardiac vagus (Beccari, 1933, 1934a and b), and on synaptic transmission in the autonomic nervous system generally (Bülbring and Burn, 1942). The antagonism of atropine to the inhibition produced by nicotine in the presence of adrenaline accords with the concept that inhibition is mediated through the parasympathetic system, as Amsler (1920) proposed. The effectiveness of hexamethonium suggests that nicotine, in the presence of adrenaline, acts at the ganglionic level. The greater effectiveness of hexamethonium in preventing stimulation by nicotine than in preventing inhibition by nicotine in the presence of adrenaline

is not in disagreement with the fact that even autonomic ganglia are not all equally sensitive to blocking agents (Paton and Zaimis, 1952). The prevention by ephedrine of the inhibitory effect of nicotine in the presence of adrenaline may be explained by assuming that ephedrine–adrenaline competition also takes place at the ganglionic level. We have observed, however, that ephedrine also prevents the inhibition caused by nicotine alone in fresh auricles. This may indicate that: (*a*) in antagonism by ephedrine, competition with adrenaline for common receptors is not, or is not solely, involved (for discussion of what is presumably a direct action of ephedrine on the cardiac vagus see Beccari, 1934a and b); (*b*) adrenaline or sympathin-like substances liberated by nicotine are always somehow implicated in the inhibitory action of nicotine. Small quantities of sympathin are produced even by normal unstimulated hearts (Külz, 1928). Thus sympathin could increase the sensitivity of parasympathetic ganglia to nicotine in fresh auricles as adrenaline does in old preparations. It is possible that the initial inhibitory action which has been described for small doses of adrenaline in isolated hearts, whether treated with ACh or not (Kolm and Pick, 1920; Chiò, 1928; Martini, 1932, in amphibia; Peruzzi, 1950, in mammals; Lands, 1949, in intact animals), may be determined by a similar mechanism acting in presence of a drug, an experimental condition, or a degree of vagal tone, which causes a discharge in parasympathetic ganglia. The prevention of this adrenaline inhibitory action by atropine has indeed been described (Kolm and Pick, 1920; Martini, 1932; Lands, 1949).

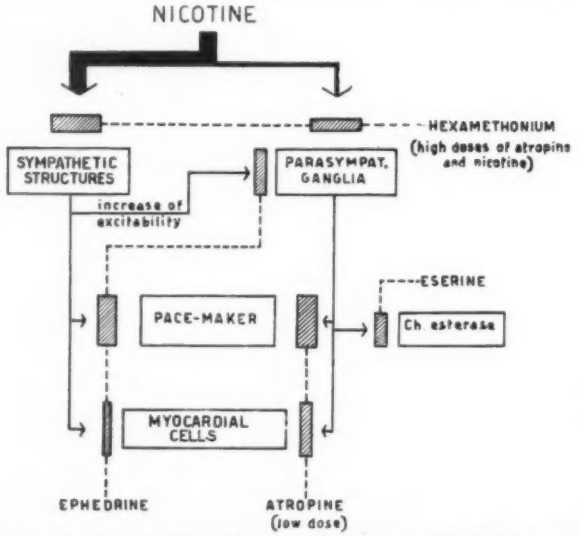

FIG. 10.—Diagram illustrating presumable sites and intensities of action of various drugs on guinea-pig isolated auricles. The action of nicotine varies qualitatively, according to the state of the auricles. A possible interpretation is: *Fresh auricles.*—The high excitability of parasympathetic ganglia, and the high activity of cholinesterase, leads to inhibition in the first phase (owing to the shorter latency of ACh) and to stimulation in the second phase (due, presumably, to sympathins and favoured by the hydrolysis of ACh). *Hypodynamic auricles.*—The low excitability of parasympathetic ganglia, and the increased sensitivity of the pace-maker and myocardial cells to sympathins, leads to predominance of stimulation. *Adrenaline-treated hypodynamic auricles.*—The restored excitability of parasympathetic ganglia (low cholinesterase activity?) results in a predominance of the inhibitory effect on frequency.

Fig. 10 is a diagram in which an attempt has been made to localize the actions of the drugs used in these experiments, and to represent, though only approximately, their different intensities of action on various auricular effector cells. In the legend to the figure the different responses to nicotine of fresh and hypodynamic auricles, and of auricles treated with adrenaline, are explained chiefly as the resultant of a progressive decrease of excitability of parasympathetic ganglia. Bülbring and Burn (1949) have shown that the power of exhausted auricles to synthesize ACh is decreased; it is not improbable that the synthetic power of parasympathetic ganglia is also impaired for, as nervous structures, they are certainly not more resistant to exhaustion than are myocardial cells. There are insufficient data to indicate whether the liberation of sympathin also decreases during exhaustion; but if this happens it would also contribute to the decrease in excitability of the parasympathetic ganglia.

SUMMARY

1. Nicotine (2×10^{-7}–2×10^{-6}) has a complex action on isolated guinea-pig auricles, causing stimulation and inhibition according to the experimental conditions.

2. The inhibitory action on rate and amplitude of contractions is evident in fresh auricles, and is prevented by atropine (2×10^{-7}), and potentiated by eserine (2×10^{-8}); the stimulant action is unmasked by atropine (2×10^{-7}) and prevented by ephedrine (5×10^{-5}); both stimulation and inhibition are prevented by hexamethonium (2×10^{-5}) and atropine (1×10^{-5}).

3. In hypodynamic auricles the nicotine inhibition of rate and amplitude is less intense than in fresh preparations, or is replaced by almost pure stimulation; adrenaline restores inhibition. The inhibitory action of nicotine in the presence of adrenaline is prevented by ephedrine, atropine and hexamethonium.

4. Hexamethonium is approximately 100-fold more effective in preventing the stimulation by nicotine of fresh atropinized auricles or of hypodynamic untreated auricles than it is in preventing

the inhibition by nicotine in the presence of adrenaline. Concentrations of hexamethonium which completely prevent the inhibition from adrenaline + nicotine do not prevent that from adrenaline + acetylcholine.

5. Injected adrenaline is, and sympathin—whether released normally or by nicotine—may be, of fundamental importance in regulating the excitability of parasympathetic ganglia.

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IMPROVED AUTOMATIC APPARATUS FOR PHARMACOLOGICAL ASSAYS ON ISOLATED PREPARATIONS

BY

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Every pharmacological assay on isolated plain muscle involves certain routine procedures such as emptying and refilling the isolated organ bath, adding one or more drugs to the bath, recording the contraction on a moving drum and allowing an interval for recovery of the tissue. Automatic apparatus for performing these various repetitive manipulations has been described before and has been in use in this and other laboratories for some time (Schild, 1946, 1947; Bülbring, 1949; Gaddum and Lembeck, 1949; Godfrey, Mogey, and Taylor, 1950; Bovet and Virno, 1952). We have found apparatus of this kind especially useful in assays involving the repetitive administration of a limited number of doses (three- and four-point assays) and in experiments in which the activities of antagonistic and potentiating drugs are measured; these drugs often produce irreversible effects and it is important that their actions be assessed against the background of a steady preparation.

In the apparatus previously described (Schild, 1946, 1947) the filling and emptying of the isolated organ bath was automatically controlled; but the drug had to be injected by hand, and the relative duration of the various stages of the assay cycle was fixed. We now describe a more adaptable, fully automatic apparatus, which can be used for most types of assay work on isolated preparations. The duration of each stage of the cycle is independently variable and can be adjusted to the response of the tissue; the assay solutions are added automatically in a predetermined but adjustable order; and an antagonistic or potentiating drug may be introduced at some stage in the cycle. The principle of controlling the flow of liquid by compressing rubber tubing with modified telephone relays, previously described, is retained in the present apparatus; but control is now on the air flow into the vessels containing the drug and washing solutions, so that the solutions do not come in contact with rubber tubing. They are rapidly brought from room temperature to the temperature of the bath as they flow through a thin-walled, jacketed warming tube. A jacketed isolated organ bath, which can be as small as 1 ml., and which is completely emptied before adding the drug solution, is used. In this way small quantities of dilute solutions may be assayed.*

PRINCIPLE OF OPERATION

The schematic diagram of Fig. 1 shows the manner in which solutions are stored and warmed and the method of emptying and filling the isolated organ bath. A and E represent electromagnetic valves which compress rubber tubing. When A is opened, air enters the reservoir B and Ringer or drug solution flows out through the jacketed warming tube C into the isolated organ bath D. When valve E is opened the solution flows out of the bath. Six reservoirs are provided, four containing active drug and two Ringer solution or an antagonistic or potentiating drug. These solutions are not diluted further before reaching the tissue and the reservoirs must therefore contain the drugs in their final dilutions.

The valves are operated at predetermined but easily adjustable intervals in such a way that the drug is brought in contact with the tissue for the required time and then removed by repeated emptying and refilling of the bath with Ringer solution. The timing of the operations is controlled by a switching unit. Impulses sent out by an electronic timer energize the driving magnets of a uniselector causing the wipers to move on to the next contact. At certain contacts of the uniselector new values of variable condensers and resistances in the timing circuit are switched in, thus altering the length of time that elapses before the next impulse is sent out. The contacts on the uniselector are wired to the valves in such a way that a complete assay cycle is carried out for each half revolution of the uniselector. Two sets of controls are provided, one to determine the duration and sequence of various steps in the assay cycle and the other to determine the sequence in which drugs are introduced

* This apparatus is now made by Casella (Electronics) Ltd.

into the bath (dose cycle). The dose cycle is controlled by a second uniselector.

Assay Cycle.—The assay cycle is divided into seven stages:

1. Emptying of isolated organ bath.
2. Refilling of bath with Ringer solution.
3. First resting period.
4. Second resting period.
5. Emptying of isolated organ bath.
6. Refilling of bath with drug solution.
7. Drug contact period.

The duration of each stage can be independently varied, that of stage 1, 2, 5 and 6 between $\frac{1}{2}$ and 500 sec. and that of stage 3, 4 and 7 between $2\frac{1}{2}$ and 2,500 sec.

This basic cycle can be modified in various ways. The main modifications are:

1. The washing of the tissue with Ringer solution (stage 1 and 2) can be repeated several times.

2. An antagonistic or potentiating drug can be introduced after stage 3 and left in contact with the tissue during stage 4.

3. Drugs can be administered by hand. A concentrated drug solution is introduced with a syringe, after adjustment of the bath level by an automatic overflow (stages 5 and 6 omitted); or a diluted drug solution is added by pipette through the warming tube (stage 6 omitted).

The rotation of the kymograph is automatically controlled. It can be switched on for a variable part of the cycle.

FIG. 1.—Diagram illustrating the manner in which drug and Ringer solutions are stored, and warmed whilst flowing into the isolated organ bath. A and E, electromagnetic valves. B, reservoir for Ringer or drug solution. C, jacketed warming tube. D, jacketed organ bath. For further explanation, see text.

Dose Sequence.—A sequence of 24 doses of 4 different drug solutions can be automatically administered in any desired order depending on the position of 24 wander plugs on the control panel. The dose-order circuit has been primarily designed for four-point assays, employing randomized sequences of four drug solutions, but a completely random sequence of 24 doses, or a systematic sequence, may be adopted. It is also possible to exclude one, two or three drug reservoirs and limit the sequence to the remainder: thus a single drug may be administered indefinitely. The present circuit could be readily modified to control 6 or more drug solutions.

Control Panel.—Table I describes in detail the various modifications of the cycle which can be achieved according to the setting of the switches on the control panel.

TABLE I

SETTING OF SWITCHES ON CONTROL PANEL TO MODIFY ASSAY CYCLE

Switch			Operation
Type	No.	Position	
Rotary	1	1 to 4	Duration of bath outflow after drug
,,	2	1 to 4	,, ,, Ringer inflow
,,	3	1 to 4	,, ,, 1st resting period
,,	4	1 to 4	,, ,, 2nd ,, ,,
,,	5	1 to 4	,, ,, outflow before drug administration
,,	6	1 to 4	Duration of drug inflow
,,	7	1 to 4	,, ,, ,, contact
,,	8	1	Independent activation of Ringer inflow valve
		2	Interruption of assay cycle
		3	Normal cycle
		4	Independent activation of bath outflow valve
,,	9	1	Bath emptied and refilled with washing solution between resting periods
		2	Bath emptied and refilled with antagonist between resting periods
		3	Resting periods combined
		4	2nd resting period by-passed
,,	10	1	Bath rinsed once
		2	,, ,, twice
		3	,, ,, three times
,,	11	1	Bath emptied and refilled automatically with dilute drug solution
		2	Bath emptied and refilled by hand with dilute solution through warming tube
,,		3	Bath not emptied. Concentrated drug added by hand
		4	Bath not emptied. Concentrated drug added by automatic syringe
,,	12	1	Drum rotating during stages 5 to 7
		2	,, ,, ,, ,, 4 ,, 7
		3	,, ,, ,, ,, 3 ,,
		4	,, ,, continuously
Push Button	13	—	Cycle uniselector advanced one step
,,	14	—	Dose-order uniselector reset to beginning of drug sequence
Toggle	15	—	1st drug reservoir excluded from sequence
,,	16	—	2nd ,, ,, ,, ,, ,,
	17	—	3rd ,, ,, ,, ,, ,,
	18	—	4th ,, ,, ,, ,, ,,

In addition to the switches listed in the table the control panel contains:

(a) seven linear potentiometers to provide fine adjustment for switches 1 to 7.

(b) A switch board containing 24 wander plugs to determine the drug sequence. Four sets of 6 plugs, each corresponding with the four drug solutions, can be plugged into sockets to produce any desired order of doses.

CONSTRUCTION OF APPARATUS

Isolated Organ Apparatus.—The isolated-organ bath, warming tube and solution reservoirs are shown in detail in Fig. 2. The particular arrangement illustrated has been designed for the repeated addition of four different drug solutions. It is important that these solutions be added at a uniform rate and that they should reach the bath by a final common path so that systematic errors are avoided.

The drug solutions are stored in vessels fitted with ground glass stoppers which carry air-inlet tubes. When the valve compressing the rubber tubing attached to the air-inlet tube is energized, air enters the reservoir and the solution flows out through a capillary tubing. Care has been taken to keep the hydrostatic pressure and the resistance of the outflow tube constant. The air-inlet tube extends nearly to the bottom of the vessel, producing a hydrostatic pressure independent of the liquid level in

the reservoir. The dimensions of the air inlet and capillary outlet tubes of each of the drug reservoirs are similar. The ends of the delivery tubes have a ground glass surface making contact with the inside of the funnel at the top of the warming tube; surface tension effects are thereby minimized and the solutions flow into the warming tube at similar rates. The ground glass tips are bevelled in such a way that the flow is not impeded (inset of Fig. 2).

The vertical warming tube consists of a thin-walled glass tube about 3 mm. in diameter surrounded by a jacket through which warm water circulates. If the tube is quite clean the solutions flow down the walls in a thin film and rapid heating is obtained. A tube 20 cm. long with a jacket temperature of 38° C. can warm a flow of 10 ml./min. from 20° to 36° C. The warming system has a negligible hold-up so that there is no mixing of one solution with another.

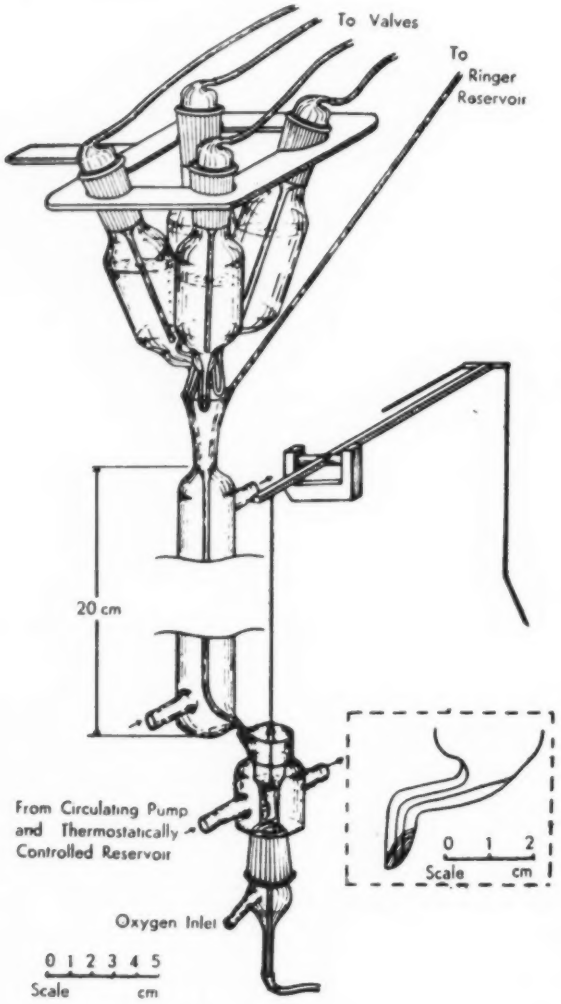

FIG. 2.—Drug reservoirs, warming tube and isolated-organ bath. Inset illustrates the bevelled end of the drug delivery tube. For explanation, see text.

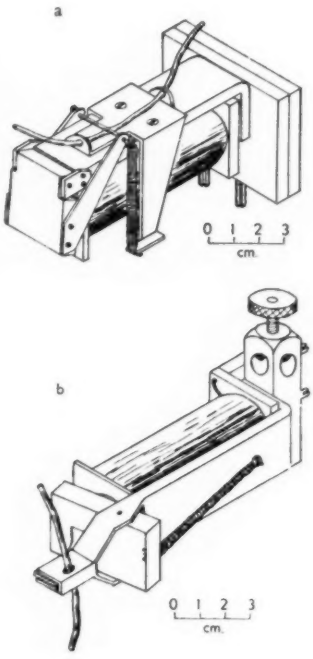

FIG. 3.—Modified telephone relays used to compress rubber tubing. Valve (a) is used on the air-inlet tube. Valve (b) has a small hold-up and is used for the bath outlet. Resistance of coils = 300 ohms. When the coils are energized the valves open.

The isolated organ bath itself is in the form of a jacketed tube about 7 mm. in diameter and 20 mm. long. This is suitable for small preparations such as the guinea-pig ileum. A bath 12 mm. in diameter and 50 mm. long has been used for bulkier preparations. The top is enlarged slightly and has an indentation to receive the end of the heating tube. The bottom is closed by a ground glass joint which carries the liquid outlet, oxygen inlet and a bridge-piece to which the lower end of the tissue is attached.

Valves.—These operate on the principle of compressing rubber tubing by means of modified telephone relays. This simple method of controlling flow has been found to be entirely satisfactory if thin-walled surgical quality tubing of 1 to 2 mm. bore is used. The valves are easily made; the type illustrated in Fig. 3a is used for controlling the air inlet of the solution reservoirs; the type in Fig. 3b is used on the liquid outlet of the bath.

Control Circuits.—The electrical part of the apparatus can be divided into three circuits—the electronic timer the cycle uniselector circuit controlling the various operations of the assay cycle, and the dose-order circuit.

The circuits are described in detail in Figs. 4 and 5 and the accompanying legends. The timer circuit is shown in the upper part of Fig. 4. It utilizes the first two banks of the cycle uniselector. The circuit was designed by Mr. V. Attree. The cycle circuit utilizes the third, fourth and fifth banks of the uniselector as shown in the lower part of Fig. 4. The third bank of contacts is wired to indicator lights, one for each stage of the assay cycle. The fourth bank controls eight electromagnetic valves regulating flow—namely, bath outlet;

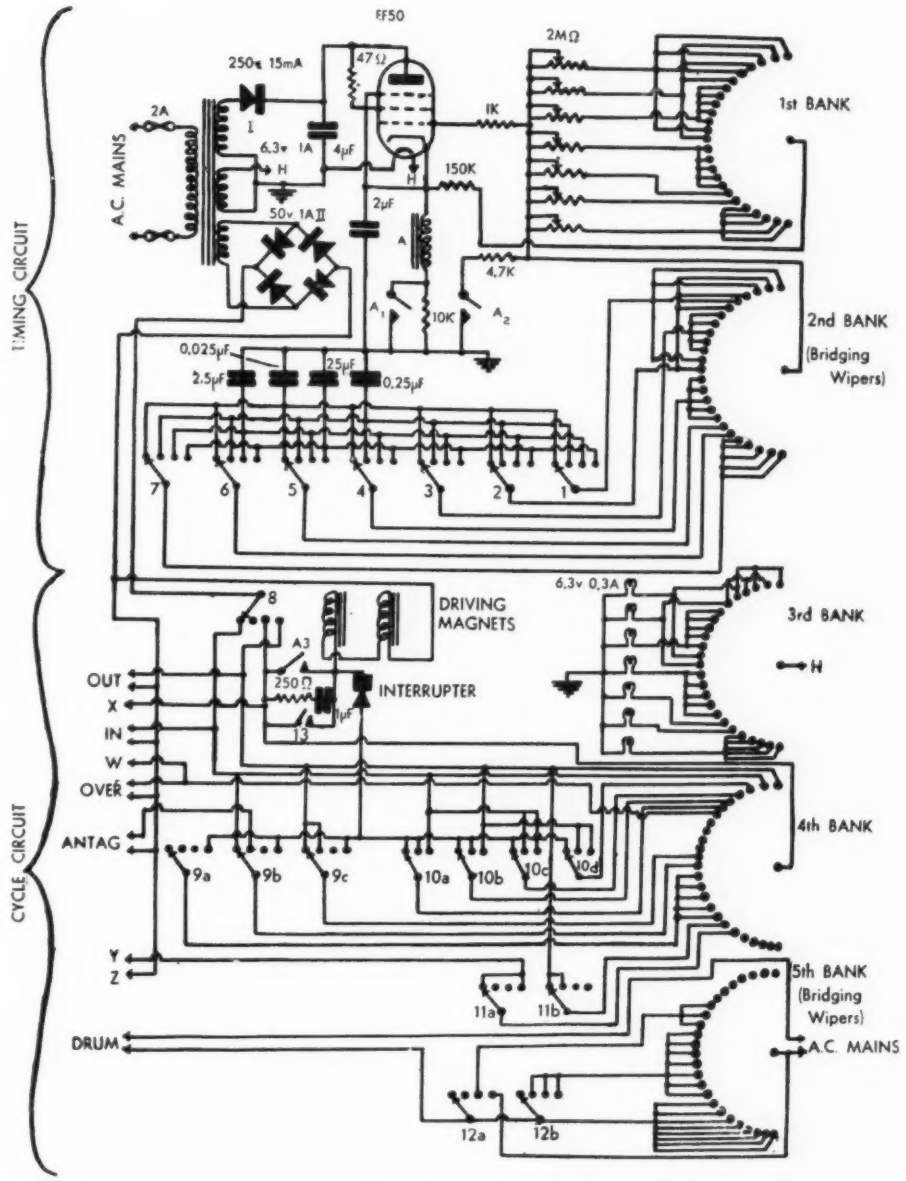

FIG. 4.—Timing and assay cycle circuit. The timing circuit occupies the first two banks of the uniselector and the assay circuit the last three banks. A standard Post Office uniselector (type 89A fitted with bank contacts, type 80) with two driving magnets each of 37.5 ohms resistance, is used. Relay A (P.O. type 3,000, coil resistance 20,000 ohms) should be so adjusted that A_1 closes before A_2 and A_3; by shorting the 10K resistance, contacts A_1 cause the relay to snap over rapidly, thus reducing sparking on A_3. Rectifiers I and II are Standard Telephones and Cables type S.B.3 and B.45–4–1FW, respectively. All resistances are rated at $\frac{1}{2}$ watt. All condensers are 350 V.W. low loss paper condensers except the smoothing condenser ($4\mu F$) which may be of the electrolytic type. OUT—Connexion to outflow valve of isolated organ bath. IN—Connexion to inflow valve of Ringer solution. OVER—Connexion to overflow valve controlling constant volume of bath. ANTAG.—Connexion to antagonist or potentiator inflow valve. DRUM—Connexion to kymograph. W, X, Y and Z connect to corresponding points in the drug selector circuit. Numbers correspond to switches in Table I.

washing solution inlet; bath overflow; to adjust the bath level; antagonist or potentiating solution inflow; and inflows for four drug solutions. The valves are activated in the sequence shown in Table II. It will be seen that the 25 steps of the cycle uniselector are subdivided into 3 long periods of 5 steps each, comprising the drug contact and the two resting periods and 10 short periods of 1 step. The fifth bank of contacts is used to switch on the drum at a suitable point in the assay cycle and to switch it off when the bath is emptied of drug solution. The

TABLE II
SEQUENCE OF OPERATIONS IN THE ASSAY CYCLE

Period:	1st, 2nd & 3rd washing						1st resting period					Additional washing		2nd resting period					Drug added		Drug in contact with tissue				
Stage No:	1	2	1	2	1	2	3	3	3	3	3	1	2	4	4	4	4	4	5	6	7	7	7	7	7
Selector contact:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Operation performed:	Bath emptying	Washing solution flowing in	Bath emptying	Washing solution flowing in	Bath emptying	Washing solution flowing in	(Overflow valve operated if needed)					Bath emptying	Washing or antagonist solution flowing in						Bath emptying if total replacement technique used	One of 4 drug solutions flowing in					

dose-order circuit is shown in Fig. 5. It employs a second uniselector which normally advances one step for each assay cycle. When certain drugs are switched out of the circuit the corresponding contacts are jumped by using the interrupter on the uniselector.

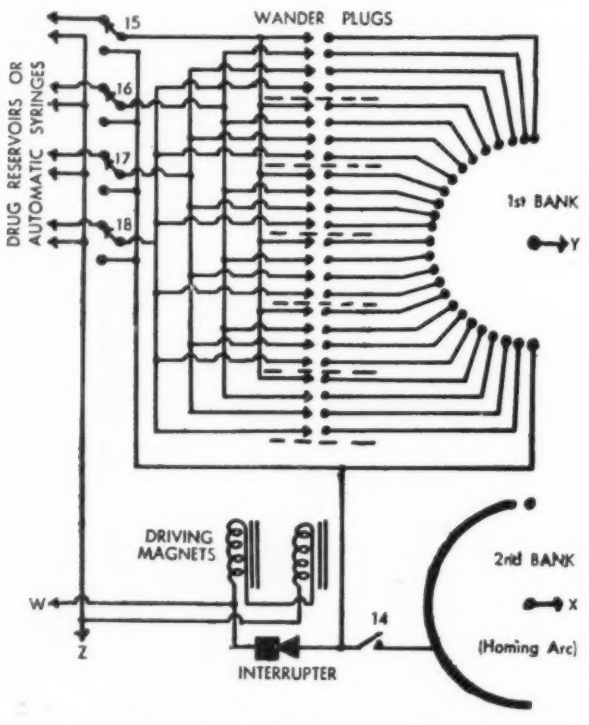

FIG. 5.—Dose-order circuit. One bank of the uniselector (P.O. type 42A fitted with bank contacts No. 42, each coil of 37.5 ohms) is connected to the valves controlling the 4 drug solutions, or to automatic injection syringes. The second bank is a homing arc which returns the uniselector to the first contact. Numbers correspond to switches in Table I.

USE OF APPARATUS

As an example of the use of the apparatus, its application to the assay of dilute solutions of histamine on the guinea-pig ileum will be described. The control panel of the switching unit is set up as follows:

Stage 1.	Emptying and draining of bath	2–3 sec.
2.	Refilling with Ringer solution	5–6 sec.
3.	First resting period	15–30 sec.
4.	Second resting period ..	by-passed
5.	Emptying of bath before addition of drug	1 sec.
6.	Refilling with drug solution ..	4–5 sec.
7.	Drug contact period	15 sec.

If the interval between operations 5 and 6 is kept as short as possible, spontaneous contractions during the change-over are usually negligible and do not interfere with the assay. The contraction after the drug has been introduced takes 12 sec. to reach a maximum; a drug contact period of 15 sec. is therefore sufficient. Switch 10 is set for two successive washings. The whole cycle thus takes about one min. The kymograph is started 5 sec. before stage 5 and stopped at the end of stage 7 (S 12–1).

Having set the assay cycle the apparatus is left running, using solutions of $2\frac{1}{2}$, 5, 10, and 20×10^{-9} histamine. After 30 to 60 min. the responses have become regular, sensitivity is at a maximum and

the preparation is ready for use. The approximate concentrations of the test solutions, diluted if necessary to lie within the range of sensitivity of the preparation, are determined by adding with a pipette through the warming tube 1 ml. of each solution in place of a standard histamine solution (S 11–2). If the response lies within the range covered by the standard solutions the concentration can be estimated by interpolation.

TABLE III

SUMMARY OF HISTAMINE ASSAY RESULTS

(a) Activity of test solutions unknown
(b) " " " " known
(c) Solutions used repeatedly

N	Dose Interval (secs.)	$\left(\frac{U}{S}\right)$ Assay	$\left(\frac{U}{S}\right)$ Theory	Actual Error	Limits of Error (P=0·05)	$\frac{s}{b}$
			(a)			
5	38	1·066	—		6·2%	·027
3	38	1·316	—		4·7%	·031
3	38	1·257	—		7·0%	·045
3	38	1·300	—		8·7%	·056
3	38	1·279	—		4·1%	·027
5	37	1·055	—		3·4%	·015
5	36	1·075	—		2·3%	·010
5	37	1·055	—		3·4%	·014
5	37	1·081	—		2·8%	·012
5	37	1·090	—		3·4%	·016
5	37	1·027	—		4·5%	·019
3	39	1·167	—		10·0%	·063
3	39	1·085	—		3·7%	·025
3	39	1·216	—		9·4%	·060
3	39	1·088	—		5·5%	·036
3	39	1·109	—		3·9%	·026
3	40	1·028	—		5·9%	·038
3	45	1·253	—		12·8%	·071
3	45	1·089	—		5·9%	·038
5	47	1·161	—		5·5%	·024
5	52	1·194	—		4·1%	·018
			(b)			
5	39	1·257	1·250	+ 0·6%	2·3%	·010
5	39	1·267	1·250	+ 1·4%	2·9%	·012
5	39	1·318	1·250	+ 5·4%	4·2%	·018
5	39	1·226	1·250	− 1·9%	4·2%	·018
5	39	1·232	1·250	− 1·4%	2·0%	·009
5	38	1·286	1·250	+ 2·9%	7·4%	·032
5	38	1·294	1·250	+ 3·5%	7·5%	·032
5	40	1·253	1·250	+ 0·3%	10·5%	·045
5	58	1·213	1·250	− 3·0%	4·7%	·020
5	55	1·268	1·250	+ 1·4%	12·1%	·052
5	45	1·257	1·250	+ 0·6%	3·5%	·015
5	45	1·254	1·250	+ 0·3%	8·1%	·035
5	45	1·257	1·250	+ 0·6%	4·0%	·017
5	45	1·222	1·250	− 2·2%	5·9%	·025
5	45	1·228	1·250	− 1·8%	3·5%	·015
5	38	1·297	1·250	+ 3·8%	4·0%	·017
5	38	1·233	1·250	− 1·4%	18·3%	·078
5	45	1·280	1·250	+ 2·4%	7·3%	·031
5	38	1·234	1·250	− 1·3%	6·8%	·029
5	38	1·246	1·250	− 0·3%	3·7%	·016
5	40	1·243	1·250	− 0·6%	6·4%	·028
5	40	1·047	1·053	− 0·6%	4·2%	·018
5	41	1·400	1·250	+12·0%	7·2%	·031
5	42	1·247	1·250	+ 0·3%	4·4%	·019
			(c)			
3	52	1·123	—		19·5%	
5	52	1·132	—		15·0%	
5	47	1·185	—		8·3%	
3	38	1·308	1·250	+ 4·6%	15·3%	
5	38	1·315	1·250	+ 5·2%	13·6%	
5	38	1·332	1·250	+ 6·6%	9·5%	
3	42	1·212	1·250	− 3·0%	11·5%	
5	38	1·278	1·250	+ 2·2%	9·9%	

For an accurate estimate, with known limits of error, a four-point assay may be used (Schild, 1942). With the information gained from the preliminary assay, two dilutions of the test solutions are prepared of approximately the same activity as the two standard solutions with which they are to be compared. It is convenient to have a ratio of 2:1 between the higher and lower concentrations of both the test and standard solutions. These four solutions are filled into the four drug reservoirs. With the dose-order plugs arranged to give successive random sequences of the four doses, the apparatus is left running till 16 to 24 contractions have been obtained. The first four contractions are generally omitted when calculating the result.

The accuracy of such an assay is high for a biological method; routine measurements of solutions containing histamine released from tissues gave limits of error of 3 to 10% (P=0.05). The average value was about 6%, corresponding to a standard deviation of about 2.5% (Table IIIa). Solutions of standard histamine assayed as "unknowns" gave similar limits. The actual deviations from the true results were known in this instance. They averaged 2.1% (Table IIIb). The details of 53 unselected assays are given in Table III, which includes the index of precision (s/b) of each experiment. The harmonic mean of s/b (Gaddum, 1953) is 0.022. A typical assay is shown in Fig. 6 and its analysis of variance in Table IV.

When only small volumes of solution are available, insufficient for an ordinary assay, it is still possible to perform a fairly satisfactory assay by collecting the solutions after they have been in contact with the tissue and re-using them. The errors of an assay of this type are greater, because the solutions are gradually altered by diffusion in and out of the tissue, and by dilution with the washing solution still in contact with the tissue and organ bath. Nevertheless, the solutions can be

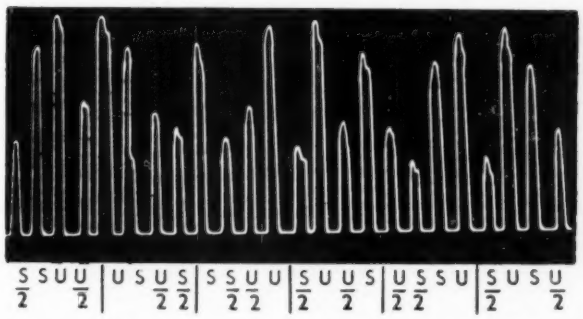

FIG. 6.—Typical four-point assay of histamine on guinea-pig ileum using the automatic apparatus.

TABLE IV

ANALYSIS OF FOUR-POINT ASSAY SHOWN IN FIG. 6

Dose	1	2	3	4	5	6	
S/2	36	41	37	35	31	30	210
U/2	52	49	51	45	44	42	283
S	80	77	78	74	73	70	452
U	90	91	86	87	83	85	522
Group totals ..	258	258	252	241	231	227	1,467

Concentration of histamine solutions:
$S/2 = 4 \times 10^{-9}$ $S = 8 \times 10^{-9}$
$U/2 = 5 \times 10^{-9}$ $U = 10 \times 10^{-9}$

True relative potency $\frac{U}{S} = 125\%$

Estimated relative potency = 123%

Limits of error from analysis of variance ($P = 0.05$) 119·5% to 126·4%.

ANALYSIS OF VARIANCE

Source	Sum of sqs.	d.f.	Mean sq.
Between groups ..	1,150	5	230
Between S & U	852	1	852
Regression	9,640	1	9,640
Parallelism	0·4	1	0·4
Error	49	15	3·3

used three or four times and still provide a reliable assay. The average limit of error of 8 assays done in this way (Table IIIc) was 13%.

SUMMARY

An automatic apparatus for pharmacological assays has been described which can be used for most types of assay on isolated preparations. All the operations of the assay cycle, such as adding and removing the drug solution, washing the tissue and controlling rotation of the drum, are performed automatically. The duration of each stage of the assay cycle can be varied independently, and the sequence in which several drug solutions are added to the tissue can be set to give any desired order.

Results obtained in 53 standard four-point assays of histamine on the guinea-pig ileum are given.

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IDENTIFICATION OF THE STABLE ANTIDIURETIC SUBSTANCE ("STABLE ADS") OF SERUM WITH 5-HYDROXYTRYPTAMINE

BY

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(RECEIVED JULY 28, 1953)

Rat serum contains, according to Ginsburg and Heller (Ginsburg and Heller, 1951; Heller, 1952; Ginsburg and Heller, 1953), two antidiuretic factors. The first, called "unstable antidiuretic substance" ("unstable ADS"), disappears on standing and is most abundant in heart or jugular serum—that is, in serum obtained from blood returning from the head: its posterior pituitary origin seems probable. The second factor, provisionally called "stable antidiuretic substance" ("stable ADS"), is not inactivated on standing, is found in arterial serum, seems to originate during coagulation—the same amount of plasma tested by the same method did not modify urine flow significantly—and to manifest its antidiuretic action only when injected subcutaneously (Ginsburg and Heller, 1953): its origin and nature are uncertain.

On the basis of unpublished observations, Ginsburg and Heller (1950) reported that they were unable to identify the "stable ADS" with 5-hydroxytryptamine (serotonin, enteramine).

This seemed surprising, since all the characteristics of the "stable ADS" noted above are shared by 5-hydroxytryptamine. Moreover, rat serum, in which "stable ADS" was first found, contains quantities of 5-hydroxytryptamine which are sufficient to influence the diuresis of hydrated rats. In fact the average 5-hydroxytryptamine content of rat serum is 0.97 μg./ml. (Erspamer and Faustini, 1953) and the minimum antidiuretic dose of this substance, by the subcutaneous route, is 0.4 μg. per 100 g. of body weight (Erspamer and Ottolenghi, 1953).

When given intraperitoneally 5-hydroxytryptamine is 5 to 10 times less effective than when injected subcutaneously (Erspamer and Ottolenghi, 1953). Ames and Van Dyke (1952) found that 4 or 6 μg. serotonin given intravenously had no antidiuretic effect in rats.

The experiments now to be described were designed to determine whether 5-hydroxytryptamine is identical with Ginsburg and Heller's "stable ADS."

METHODS

Blood was obtained by decapitation, except in dogs and in man, when it was taken from the femoral artery or from the antecubital vein. After collection it was kept for 2 to 3 hours at room temperature and then for a further 15 to 20 hours in a refrigerator. The serum was separated by centrifugation.

Serum samples were obtained from 20 human beings, 15 rats, 10 rabbits, 3 dogs, 3 cats, 2 goats, and 10 hens.

In some experiments native serum was compared with acetone extracts of serum, but in most experiments acetone extracts only were studied. To prepare these extracts, serum was treated with 4 volumes of acetone, was left standing overnight in the refrigerator and then filtered. The solvent was evaporated under reduced pressure just before use, and was then brought to the desired volume with distilled water. The acetone extracts keep indefinitely and thus offer homogeneous material for several successive experiments.

Estimations of antidiuretic activity were made on unselected adult albino rats of both sexes, weighing from 150 to 270 g. In all, 180 groups of four animals each were used. Two doses of water, the first of 2.5 ml./100 g., and the second of 5 ml./100 g., were given by stomach tube with an interval of 3 hours.

Serum, serum extracts, 5-hydroxytryptamine solutions (Farmitalia, S.p.A.) and control saline were injected subcutaneously (1 ml./100 g. rat) immediately after the second dose of water. The urine output was measured for seven hours thereafter, at intervals of 30 to 60 min.

RESULTS

Effect of Serum and of Acetone Extracts of Serum on Water Diuresis.—In rats and rabbits we have made separate assays of serum, of acetone extracts of serum, and of serum proteins precipitated by acetone and dissolved in water.

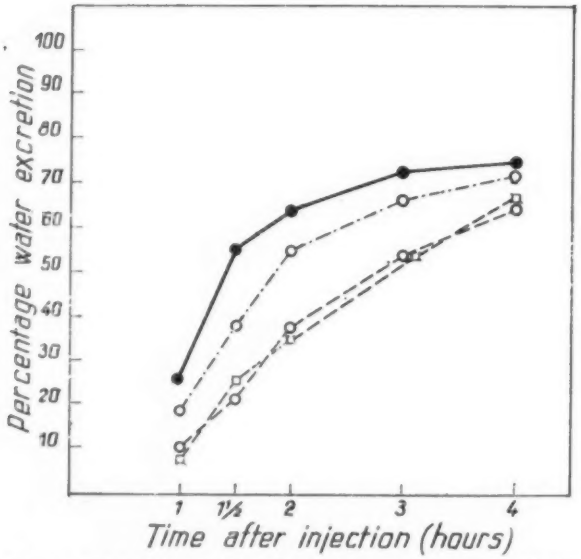

FIG. 1.—The antidiuretic effect of rabbit serum compared with that of the acetone extract of the same serum. Unanaesthetized rats. Subcutaneous injections. ●——● 1.0 ml. saline per 100 g.; ○- - -○ 1.0 ml. serum per 100 g.; ○-·-·-·-○ 0.1 ml. serum per 100 g.; □- - -□ acetone extract corresponding to 1.0 ml. serum per 100 g. Each point of the curves is the mean of results from 3 groups of 4 animals each.

Fig. 1 clearly shows that sera and their acetone extracts possess the same antidiuretic activity. The antidiuretic principle of serum has therefore passed completely into the acetone.

The moderate antidiuretic action provoked by the acetone precipitate was not further investigated, but it may be due to breakdown products of serum proteins (cf. oxytocic action of pepsitensin, as described by Croxatto, Rojas, and Barnafi, 1950).

Because of these first results only acetone extracts of serum were used in the subsequent experiments.

Antidiuretic Action of Extracts Prepared from Sera of Different Species.—Extracts of dog, cat, goat, rabbit, rat, hen, and human serum were compared with one another and with solutions of 5-hydroxytryptamine. The results are shown in Tables I and II.

Quantitative estimations of 5-hydroxytryptamine on the rat uterus (Erspamer, 1940a) gave, in terms of μg. of 5-hydroxytryptamine base per ml. of serum, the following values for the different extracts: man, 0.16; dog, 0.18; cat, 5.60; goat, 2.20; rabbit, 4.30; rat, 1.05; hen, 2.70.

Having previously demonstrated that the antidiuretic action of 5-hydroxytryptamine is only approximately proportional to the dose (Erspamer and Ottolenghi, 1953), we now find that for the majority of the sera examined there is a satisfactory agreement between antidiuretic effect and 5-hydroxytryptamine content. Extracts of cat and hen serum are exceptions; these have much less antidiuretic action than one would expect from their 5-hydroxytryptamine content.

It is difficult to account for these discrepancies. It may be that serum contains substances which retard the absorption of 5-hydroxytryptamine from the site of injection, or substances which, without modifying its absorption, interfere directly with its renal effects. Further work is necessary to clarify this point.

Tables I and II show that, of the various species tested, the extract of rabbit serum had the most powerful antidiuretic effect. Doses of this extract corresponding to 0.1 ml. of serum per 100 g. of body weight caused a significant reduction in urine flow. Extracts of rabbit serum were therefore used for experiments on inactivation and inhibition of the antidiuretic substance.

Antidiuretic Action of the Rabbit Serum Extracts after Treating with Diazonium Salts and with Guinea-pig Intestine Homogenates. — Diazonium salts (Erspamer, 1940b, 1948) and extracts of guinea-pig intestine (Erspamer, 1942) have been shown to inactivate crude enteramine extracts. The following liquids were therefore given subcutaneously to rats in doses of 1 ml. per 100 g.:

TABLE I

THE EFFECT OF SUBCUTANEOUS INJECTION OF SERUM EXTRACTS FROM DIFFERENT ANIMAL SPECIES (AMOUNTS OF EXTRACT EQUIVALENT TO 1·0 ML. SERUM PER 100 G. OF BODY WEIGHT) ON THE WATER DIURESIS OF HYDRATED RATS

Species from which Serum Extract was Prepared	No. of Rats	Percentage Water Excretion					
		1 hr.	1½ hr.	2 hr.	3 hr.	4 hr.	7 hr.
Nil (saline control)	40	29·2	54·0	62·5	73·2	78·0	93·0
Man	8	35·0	61·2	70·0	72·0	76·0	87·0
Dog	24	29·3	55·7	67·0	77·8	78·3	91·0
Cat	16	21·0	43·0	54·0	69·0	73·0	85·0
Goat	16	19·0	37·0	47·0	57·0	64·0	79·5
Rabbit ..	24	3·2	14·7	26·0	48·2	57·3	80·3
Rat	8	20·5	39·0	53·0	63·0	72·5	92·5
Hen	8	21·0	33·0	53·0	62·5	65·0	86·0

TABLE II

THE ANTIDIURETIC EFFECT OF DIFFERENT DOSES OF THE ACETONE EXTRACT OF RABBIT SERUM

	No. of Rats	Percentage Water Excretion				
		1 hr.	1½ hr.	2 hr.	3 hr.	4 hr.
Saline	40	29·2	54·0	62·5	73·2	78·0
0·05 ml. serum/100 g. ..	16	28·0	51·5	60·5	69·0	76·0
0·1 ,, ,, ,, ..	16	19·3	41·8	58·3	66·5	71·0
0·2 ,, ,, ,, ..	24	13·6	35·5	52·3	65·3	70·0
0·5 ,, ,, ,, ..	32	6·6	22·2	37·8	53·2	68·8
1·0 ,, ,, ,, ..	24	3·2	14·7	26·0	48·2	57·3
0·004 ,, mg. 5-hydroxytryptamine/100 g. ..	48	8·7	25·7	39·7	55·3	66·8

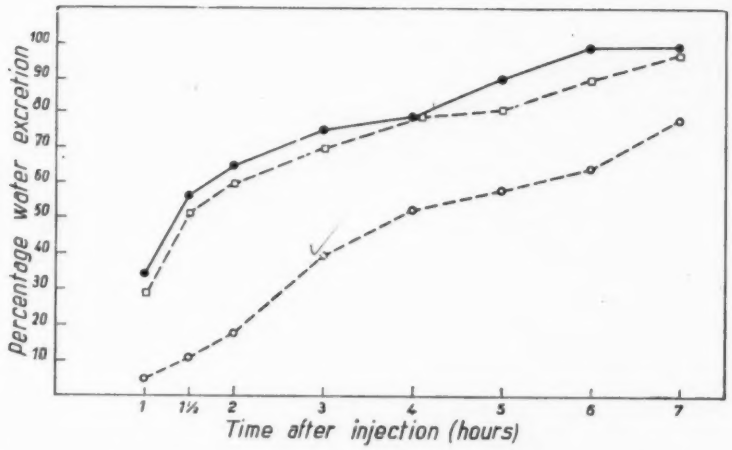

FIG. 2.—The antidiuretic effect of rabbit serum extract, 1.0 ml. per 100 g., before (○- - -○) and after (□- - -□) treating with diazotized *p*-nitroaniline. ●——● 1 ml. saline per 100 g. Each point of the curves is the mean of results from 2 groups of 4 animals each.

(a_1) *Serum Extract Treated with Diazotized* p-*Nitroaniline*.—The extract from 30 ml. rabbit serum was concentrated under reduced pressure, then brought to 21 ml. with distilled water; 4.5 ml. of diazotized *p*-nitroaniline and 4.5 ml. of 2% sodium bicarbonate were added. A reddish-yellow colour was produced.

(a_2) *Control Saline*.—21 ml. saline was substituted for the serum extract.

(b_1) *Serum Extract Treated with Homogenate of Guinea-pig Intestine*.—The extract corresponding to 20 ml. of serum was evaporated under reduced pressure and the dry residue taken up with 4 ml. of 0.07 M phosphate buffer at *p*H 7.4. After adding 4 ml. of a homogenate of guinea-pig small intestine diluted to 1/4 with phosphate buffer, the liquid was kept for 1 hour in a water bath at 39° C. and oxygen was bubbled through. 30 ml. acetone was now added. After standing in a refrigerator overnight the liquid was filtered, freed from the acetone by distillation under reduced pressure, and then brought to 40 ml. with distilled water (1 ml. liquid =0.5 ml. serum).

(b_2) *Serum Extract Treated with Guinea-pig Intestine Homogenate and Methylene Blue*.—The sample was treated exactly as above with the sole difference that the addition of the intestine homogenate was preceded by that of 1 ml. of 0.002 M methylene blue.

(b_3) *Control*.—Treated as b_1, but with saline substituted for serum extract.

Figs. 2 and 3 show the results of these experiments. It will be seen that treatment with both diazonium salts and guinea-pig intestine homogenate

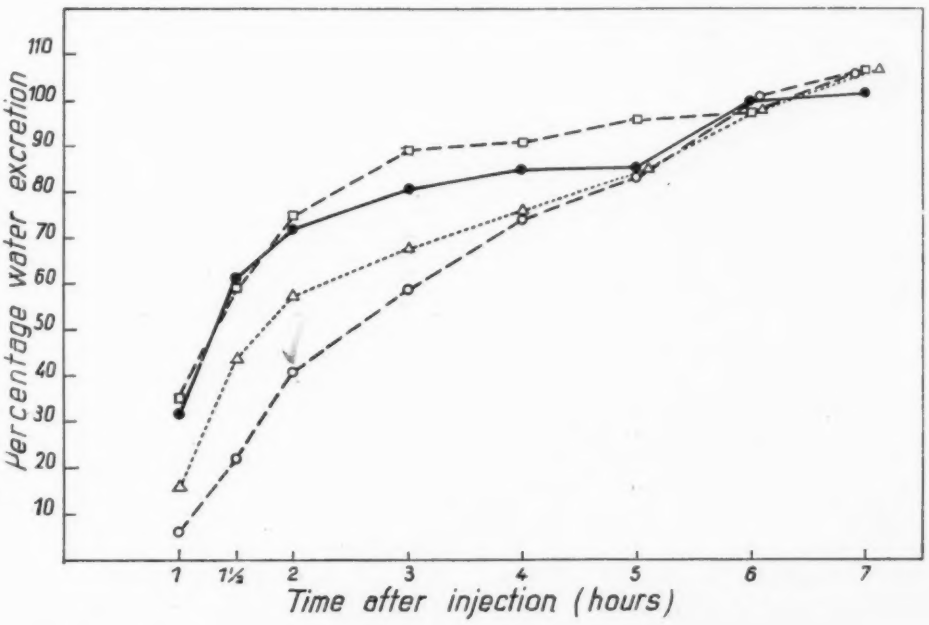

FIG. 3.—Inactivation of the extract of rabbit serum by homogenates of guinea-pig small intestine. ●——● 1.0 ml. saline per 100 g.; ○- - -○ untreated extract corresponding to 0.5 ml. serum per 100 g.; □- - -□ the same quantity of extract after incubation with homogenate of guinea-pig intestine; △.....△ the same quantity of extract after incubation with intestine homogenate + methylene blue. Each point of the curves is the mean of results from 4 groups of 4 animals each.

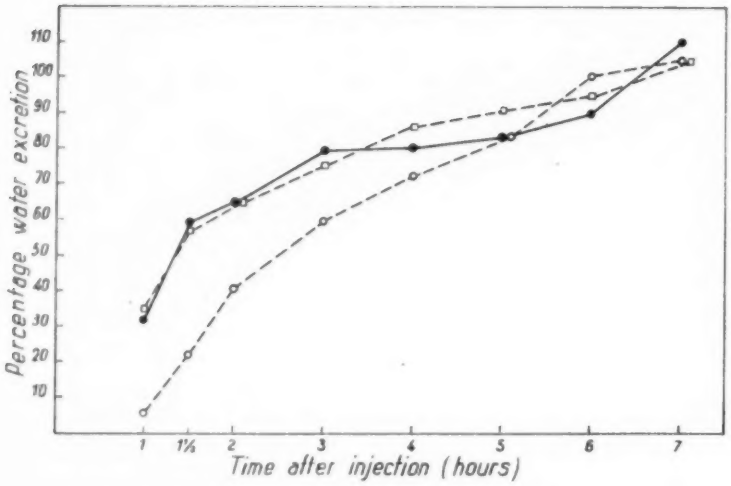

FIG. 4.—The antidiuretic effect of rabbit serum extract, 0·5 ml. per 100 g., on normal rats (○- - - -○) compared with that on rats pretreated with 10 mg./kg. of dibenamine intraperitoneally (□- - - -□). ●——● 1.0 ml. saline per 100 g. Each point of the curves is the mean of results from 3 groups of 4 animals each.

completely destroys the antidiuretic activity of the serum extracts, exactly as it destroys the spasmogenic and the antidiuretic activity of crude enteramine extracts and of pure 5-hydroxytryptamine (Erspamer, 1940b, 1942, 1948, 1953a).

It is very probable that the inactivating agent in the intestine homogenate is amine oxidase. This view is supported by the observation that the disappearance of the antidiuretic effect of serum is at least partly prevented by adding methylene blue —a known inhibitor of amine oxidase (Blaschko, 1952)—to the homogenate.

Controls carried out on the atropinized oestrous uterus of the rat showed that the liquids a_1 and b_1 had no stimulant action, while liquid b_2 had about 30% of the activity of the untreated serum extract. The incomplete inhibition of amine oxidase by methylene blue can be ascribed to the long duration of the inactivating treatment, combined with the very high content of enzyme in the guinea-pig small intestine.

Antidiuretic Effect of the Rabbit Serum Extract in Rats Treated with Dibenamine.—Dibenamine is one of the most powerful antagonists of 5-hydroxytryptamine (Erspamer, 1952, 1953b; Fingl and Gaddum, 1953). Three groups of 4 animals each were injected intraperitoneally with 0.5 ml. of 0.2% dibenamine hydrochloride solution per 100 g. of body weight, 3 hours before the administration of the rabbit serum extract (0.5 ml./100 g.). Fig. 4 shows that treatment with dibenamine produced a complete inhibition of the antidiuretic effect of the serum extract.

Influence of the Route of Administration on the Antidiuretic Effect of the Rabbit Serum Extract.—Fig. 5 shows that the subcutaneous route is much more effective than the intraperitoneal one. The decrease in urine flow was more pronounced after 0.2 ml. of serum extract given subcutaneously than after 1 ml. intraperitoneally. Doses of extract corresponding to 0.5 ml. of serum were completely ineffective by the intraperitoneal route.

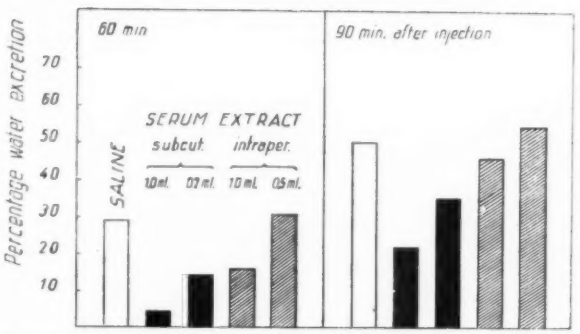

FIG. 5.—Influence of the route of administration on the antidiuretic effect of rabbit serum extract. The doses shown were given per 100 g. of body weight. Each column represents the mean of results from 2 groups of 4 animals each.

An identical behaviour has been described for 5-hydroxytryptamine by Erspamer and Ottolenghi (1953).

Preliminary Experiments on the Mechanism of the Antidiuretic Action of Serum.—Three groups of 4 rats were injected subcutaneously with a dose of extract corresponding to 1 ml. of rabbit serum per 100 g. of body weight and, at the same time but in a different site, with 0.5 ml./100 g. of a 4% solution of creatinine (200 mg./kg.). Creatinine was also administered to three control groups.

Fig. 6 shows that the extract of rabbit serum, besides reducing urinary flow, also reduces (though apparently for a shorter period) the renal excretion of creatinine. This signifies that, in the mechanism of the antidiuretic action of serum, decreased

glomerular filtration must play an important if not an exclusive part.

Since rabbit serum, given in subcutaneous doses of 2 ml./100 g. or less, does not modify the systemic blood pressure of the intact, unanaesthetized rat, we conclude that the decrease in filtration rate is due to constriction of the afferent glomerular arterioles causing a decrease in intraglomerular pressure.

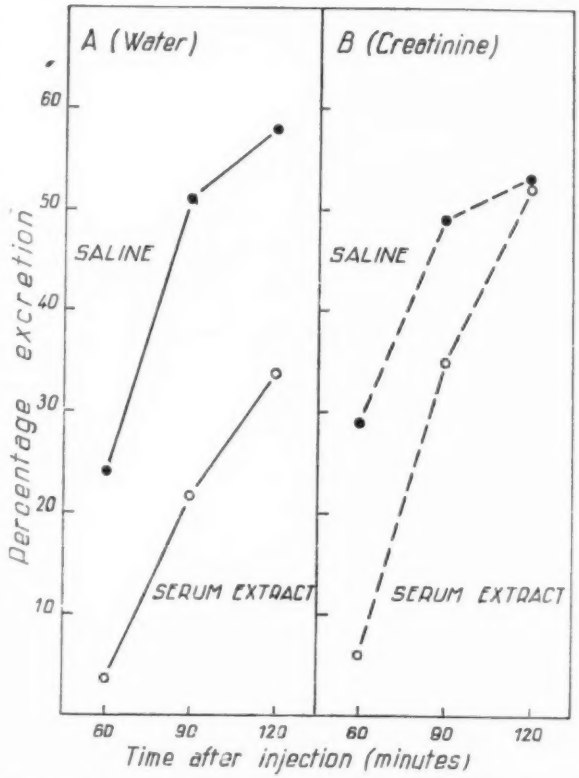

FIG. 6.—Comparison between the renal excretion of water (5 ml./100 g.) and that of creatinine (200 mg. kg.) after subcutaneous injection of extract corresponding to 0.5 ml. rabbit serum per 100 g. Each point of the curves is the mean of results from 2 groups of 4 animals each.

The vascular point of attack of 5-hydroxytryptamine in the rat kidney has been emphasized by Erspamer and Ottolenghi (1953).

DISCUSSION

The findings of Dicker and Ginsburg (1950) and of Ginsburg and Heller (1951, 1953) on the presence of a stable antidiuretic factor ("stable ADS") in rat serum were completely confirmed. Furthermore, it was demonstrated that the same factor is contained in rabbit, cat, goat, and hen serum, often in very considerable quantity.

The "stable ADS" has the following characteristics: (*a*) it is apparently present only in serum and not in plasma, since it becomes detectable by our methods only after coagulation; (*b*) it is more active after subcutaneous injection than after administration by other routes; (*c*) it may be extracted from serum by acetone; (*d*) it is completely destroyed by treating the serum with diazonium salts and with homogenates of guinea-pig small intestine (amine oxidase), but it is not attacked by serum enzymes; (*e*) it is ineffective when administered after dibenamine; (*f*) finally, it provokes antidiuresis by a decrease of glomerular filtration rate caused by an afferent vasoconstriction. All these characteristics of "stable ADS" are shared by 5-hydroxytryptamine.

It should be added that 1 ml. rat serum when given per 100 g. rat contains more than the minimum antidiuretic dose of 5-hydroxytryptamine. The antidiuretic effect of serum from different species is, with a few exceptions, satisfactorily proportional to its 5-hydroxytryptamine content, as established by bioassay on the atropinized oestrous uterus of the rat. Human serum has very little 5-hydroxytryptamine; its content per ml. is lower than the minimum antidiuretic dose per 100 g. of rat. Dog serum is similar. Rabbit serum is very rich in 5-hydroxytryptamine, and has a powerful antidiuretic action.

All these results strongly support the identity of the "stable ADS" with 5-hydroxytryptamine, and no observations exist, as far as we know, which are unfavourable to this conclusion.

From the recent work of Erspamer and Faustini (1953), the hydroxytryptamine content of the serum of more than 30 animal species, including practically all the domestic and laboratory ones, is now known. It is easy to predict, with good approximation, the antidiuretic action of these sera in hydrated rats. One can presume that only sera with a 5-hydroxytryptamine content above 0.3–0.4 μg./ml. are likely to elicit a detectable antidiuretic response.

Heller (1952) found that the "stable ADS" was usually present in rat serum, but not invariably so. The occasional failure to detect this substance may now be explained by our finding that the 5-hydroxytryptamine content of rat serum varies considerably—from 0.57 to 1.75 μg./ml. in 35 serum samples. When 1 ml./100 g. is injected subcutaneously into rats, the lower values nearly coincide with the minimum antidiuretic dose of pure 5-hydroxytryptamine.

Work is in progress to investigate the changes in the 5-hydroxytryptamine content of serum under various experimental and pathological conditions.

Summary

1. Serum and acetone extracts of serum from normal rats, rabbits, cats, goats, and hens have a marked antidiuretic effect when injected subcutaneously into hydrated rats. The presence in serum of an antidiuretic factor of non-pituitary origin ("stable ADS" of Ginsburg and Heller, 1953) is confirmed.

2. The antidiuretic factor is inactivated *in vitro* by diazonium salts and by homogenates of guinea-pig small intestine (amine oxidase), and its action is blocked *in vivo* by dibenamine. Its activity is much more conspicuous after subcutaneous injection than after intraperitoneal administration.

3. Preliminary experiments on the renal excretion of creatinine by rats show that the antidiuretic effect of serum extracts may be ascribed mainly to a reduction in glomerular filtration rate produced by constriction of afferent glomerular arterioles.

4. Since (1) the "stable ADS," like 5-hydroxytryptamine, apparently originates in serum during coagulation, (2) the two substances share many chemical and pharmacological characteristics, (3) their mechanism of renal action appears to be the same, and (4) serum from different animal species shows an antidiuretic action which is satisfactorily proportional to its 5-hydroxytryptamine content, it seems justifiable to postulate the identity of the stable antidiuretic substance, of serum and of serum extracts, with 5-hydroxytryptamine.

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Brit. J. Pharmacol. (1954), 9, 37.

THE CHEMOTHERAPEUTIC ACTION OF PHENANTHRIDINE COMPOUNDS

PART VII

THE EFFECT OF 2 : 7-DIAMINO-9-α-THIENYL-10-METHYLPHENANTHRIDINIUM CHLORIDE 621C47) UPON INFECTIONS WITH *TRYPANOSOMA CONGOLENSE* AND *T. VIVAX* IN CATTLE*

BY

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(RECEIVED AUGUST 14, 1953)

In a previous paper Brownlee, Goss, Goodwin, Woodbine and Walls (1950) described a number of new phenanthridinium compounds which were as active as dimidium against laboratory infections of *Trypanosoma congolense* in mice. Four of these compounds were subsequently given trials in cattle in Northern Nigeria (Goodwin and Unsworth, 1952; Goodwin and Chandler, 1952). One compound (150C47) was given an extended trial and the results showed that it was too toxic for use; another (621C47) eradicated the trypanosome infection and proved relatively non-toxic.

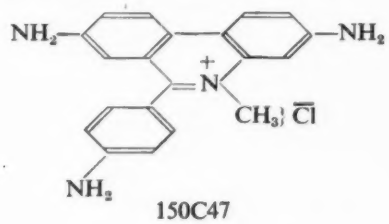
As the number of cattle used in the Nigerian experiments with 621C47 was very small, a further trial with this drug was carried out in the Sudan. A few experiments were also conducted with 150C47. A preliminary report of these experiments has been published by Evans (1952). The present paper gives the results in full.

MATERIAL AND METHODS

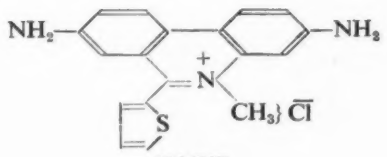
The structural formulae of 150C47 (2:7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride) and 621C47 (2:7-diamino-9-α-thienyl-10-methylphenanthridinium chloride) are shown in the next column. For comparison with these, the structural formula of dimidium is also given.

It should be noted that the bromide of 621C47, used in Nigeria by Goodwin, is less soluble than the chloride used in the present experiments. The drugs 150C47 and 621C47 were dissolved in water to give 2% w/v solutions, and doses were given subcutaneously behind the shoulder. Dimidium was given as a 1% solution and, for comparison, antrycide methylsulphate as a 5% solution—both by the subcutaneous route.

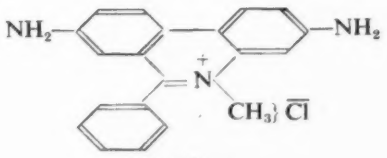
The experiments fall into two groups according to their geographical location, those carried out at Khartoum and those at Malakal (situated on the White Nile about 450 miles south of Khartoum). As the environmental conditions differed considerably, the two sets of experiments are described separately.



150C47



621C47



Dimidium

Khartoum Experiments

The animals used were bull calves, 1 to 2 years old, of the Zebu type commonly found in the Northern Sudan. Each calf was given a serial number and tethered to a stake. All were fed twice daily with freshly cut clover and unripe "*durra*" (*Sorghum*): water was also given twice daily.

The strains of *T. congolense* and *T. vivax* were originally obtained from natural infections observed in

* This work was done while R. A. Neal was working as a guest at the Veterinary Research Laboratories, Khartoum.

cattle at Malakal, and had since been transmitted by syringe. At the time of the first experiments the strain of *T. congolense* had been passaged six times in mice and twice in cattle, and that of *T. vivax* three times in cattle. In subsequent experiments the trypanosomes were drawn from positive controls of the preceding experiments. The animals were infected by subcutaneous injection of 5 ml. of infected blood.

Drug-resistant strains of *T. congolense* were obtained by treating the infected calf with sub-curative doses and, after it had relapsed, re-treating it with slightly larger amounts of drug. In this manner, two strains of *T. congolense* were obtained, one resistant to treatment with 5 mg./kg. of antrycide methylsulphate, the other resistant to 1.5 mg./kg. of dimidium.

The presence or absence of trypanosomes was determined by examining a drop of the ear blood. When it was impossible to examine fresh blood, dry films were stained with Field's stain and examined at a more convenient time. One hundred microscopic fields were examined (1/6 in. objective and 2 × ocular) before a blood-film was recorded as negative.

The blood of cattle inoculated with trypanosomes was examined daily until positive; the animals were then divided into groups and treated with the drugs. After treatment the ear blood was examined microscopically for trypanosomes as often as possible. The exact number of such examinations is recorded for each experiment.

Punch biopsies of the liver were taken in order to observe the effect of the phenanthridinium drugs upon that organ. The tissue was fixed in formol-saline. Frozen sections were cut, and stained with Sudan III.

The advantage of the experiments conducted at Khartoum is the absence there of any natural transmission of *T. congolense* or *T. vivax* by insect vectors; hence relapses after treatment could be attributed to failure of the drug treatment. As a precaution against the remote possibility of mechanical transmission of *T. congolense* or *T. vivax* by biting flies, all the cattle were sprayed with "gammexane" suspension twice weekly.

Malakal Experiments

The main difference between the experiments conducted at Malakal and those at Khartoum is that at Malakal the cattle were turned out to graze in the bush and, as the experiments were carried out during the dry season (February to April), there was very little green grazing except on the banks of the river. These conditions of poor nutrition and low water intake would be likely to reveal the toxic side-effects of a drug.

Since previous experiments in Nigeria (Goodwin and Chandler, 1952), and our experiments at Khartoum, had shown that 150C47 was too toxic for use only 621C47 was tried in the Malakal experiments.

Two groups of 30 cattle (1 to 3 year old Zebu bull calves, purchased locally) were inoculated with infected blood from naturally infected cattle—one group with *T. congolense*, the other with *T. vivax*. In addition, 6 calves found spontaneously infected with *T. vivax* were included in the group artificially infected with *T. vivax*. Not all the cattle inoculated became infected, but all were treated with 621C47.

The dose of drug used was 2 mg./kg., as the Khartoum work showed that doses lower than this were ineffective against *T. vivax*. Liver punch biopsies were taken from a few animals.

In the vicinity of Malakal no tsetse flies have been observed, but *T. vivax* and *T. congolense* are transmitted mechanically by blood-sucking flies. However, the experiments were carried out at the end of the dry season when the number of these flies is at a minimum.

As a further precaution against re-infections being confused with relapses, a small experiment was carried out to determine if 621C47 showed any prophylactic properties. A group of 50 calves was treated with 2 mg./kg. of 621C47. At weekly intervals after dosing, 5 of these animals were challenged with an inoculation of blood from a calf infected with *T. congolense*, and a further 5 were challenged with *T. vivax*. Two normal animals were also inoculated with the infected blood to act as controls at each challenge. After challenging, all cattle were examined on three successive days each week for 9 weeks.

Results

Therapeutic Experiments at Khartoum

The therapeutic effects of 621C47 and 150C47 upon *Trypanosoma vivax* and *T. congolense* are shown in Table I.

TABLE I

EFFECT OF 621C47 AND 150C47 UPON *TRYPANOSOMA CONGOLENSE* AND *T. VIVAX* INFECTION IN CATTLE

Drug	Dose mg./kg.	Cattle Infected with *T. vivax*		Cattle Infected with *T. congolense*	
		Ratio of Animals Cured to Animals Infected	Period Observed (Months)	Ratio of Animals Cured to Animals Infected	Period Observed (Months)
621C47	0·5	7/9	2·5	5/6	3
	1·0	4/8	2·5	6/6	3
	2·0	5/5	3	5/5	3
	4·0	—	—	4/4	3
—	Controls	0/6	3	0/4	0·5
150C47	0·5	0/5	4	4/6	4
	1·0	1/5	4	6/6	4
	2·0	2/2	4	6/6	4
—	Controls	0/4	4	0/7	4

These results show that a minimum dose of 2.0 mg./kg. of either 621C47 or 150C47 is required to cure all cattle infected with *T. vivax*. The animals began to relapse 13 days after treatment with 150C47 and 28 days after treatment with 621C47. In the blood of 2 of the 4 animals which relapsed after treatment with 1.0 mg./kg. of 621C47, trypanosomes were observed on 2 occasions only, but more frequently in the blood of the other 2 calves.

When it became apparent that doses of 1.0 and 0.5 mg./kg. of 621C47 were not sufficient to prevent relapses, the blood examination of these groups was stopped but the examination of those treated with 2 mg./kg. was continued. The blood of each calf in this group was examined 78 times over a period of 92 days without finding a trypanosome. Three of the infected, but untreated, calves died of acute trypanosomiasis; the others, however, survived, though trypanosomes were frequently observed in the blood up to the end of the experiment.

It should be noted that the results of treatment of *T. vivax* with 621C47 given in Table I are composite figures from three separate experiments. As they were carried out under similar conditions and gave similar results, they are treated here as one experiment.

Both of the phenanthridinium drugs were more effective against *T. congolense* than against *T. vivax*. In experiments with *T. congolense* a dose of 1.0 mg./kg. successfully eradicated the infection. Trypanosomes appeared in the blood of one calf 32 days after treatment with 0.5 mg./kg. of 621C47, and it was re-treated (30 days after observation of the relapse) with 1.0 mg./kg. of 621C47. This animal again relapsed 25 days after re-treatment; it was then given 1.5 mg./kg. and thereafter remained negative. All four control animals died of acute trypanosomiasis within 14 days after the other groups had been given the drug. The blood of the 621C47-treated cattle was examined for trypanosomes on 71 occasions over a period of 100 days.

At the end of the experiment, 20 ml. of blood was taken from each calf of the group treated with 1.0 mg./kg. of 621C47, pooled and sub-inoculated into two clean calves. These animals remained free from trypanosomes.

TABLE II

EFFECT OF 621C47 AND 150C47 UPON DRUG-RESISTANT STRAINS OF *T. CONGOLENSE*

Drug	Dose mg./kg.	Cattle Infected with Dimidium-resistant *T. congolense*		Cattle Infected with Antrycide-resistant *T. congolense*	
		Ratio of Animals Cured to Animals Infected	Period Observed (Months)	Ratio of Animals Cured to Animals Infected	Period Observed (Months)
621C47	1·0	0/3	1·5	—	—
	2·0	2/4	1·5	0/6	2
	3·0	—	—	0/6	2
Antrycide dimethyl sulphate	5·0	4/4	3·8	0/3	2
Dimidium	1·5	1/3	1·5	—	—
—	Controls	0/3	2	0/3	2
150C47	1·0	—	—	0/6	2·8
	2·0	—	—	0/6	2·8
	3·0	—	—	5/6	2·8
Dimidium	1·5	—	—	0/3	2·8
—	Controls	—	—	0/5	2·8

Table II gives the results of treatment of cattle infected with drug-resistant strains of *T. congolense*.

They show that against an antrycide-resistant strain of *T. congolense*, 150C47 was active only at a very high dose-level, while 621C47 was inactive. The calves began to relapse 11 days after treatment with 150C47, and 14 days after treatment with 621C47. On the 71st day after treatment, the surviving calves from the groups given 621C47 were re-treated with 5.0 mg./kg. of antrycide methylsulphate and 2.0 mg./kg. of 621C47 simultaneously. The blood of all six animals remained negative (examined on 36 occasions over a period of 60 days), in contrast to the control animal which was positive. One calf of the re-treated group died from unknown causes.

621C47 had very little therapeutic effect upon *T. congolense* made resistant to dimidium. However, because of the chemical similarity of these two drugs, this result was not unexpected. An interesting observation in this experiment was that the calves treated with antrycide were apparently cured (blood examined on 59 occasions over a period of 111 days).

Experiments at Malakal

Therapeutic Experiments.—Twenty-four of the 29 calves infected with *T. congolense* were dosed with 2.0 mg./kg. of 621C47, with the remaining 5 left as controls. Similarly, 15 of the 19 calves infected with *T. vivax* were given 2.0 mg./kg. of 621C47, leaving 4 infected animals as controls.

After treatment a drop of blood from each calf was examined microscopically for trypanosomes on three successive days each week for 12 weeks. All the treated cattle were negative throughout this period. The controls remained infected, and three from each control group subsequently died from acute trypanosomiasis.

Two calves of the treated *T. congolense* group died, one from pleuropneumonia and the other from an unknown cause. A third calf had to be slaughtered on account of a broken leg. In the *T. vivax* group of calves, two died of pleuropneumonia.

Prophylactic Experiments. — Calves challenged with *T. congolense* one and two weeks after dosing with 2 mg./kg. of 621C47 remained negative throughout the duration of the experiment, whereas all calves challenged three, four and five weeks respectively after dosing became infected. All ten control calves were positive.

All animals challenged with *T. vivax* one week after dosing remained negative, but two of the five calves challenged two weeks after dosing developed infections of *T. vivax*. All calves challenged three and four weeks after dosing remained negative up to the end of the experiment. Nine of the ten control animals were positive.

It is unfortunate that the results with *T. vivax* were not as uniform as those with *T. congolense*. However, *T. vivax* is notorious for its irregular behaviour.

These experiments show that 2 mg./kg. of 621C47 will protect cattle from infection with *T. congolense* for only two weeks, and with *T. vivax* for one week. This agrees with the previous experiments which showed that *T. vivax* is more resistant to treatment with 621C47 than is *T. congolense*.

Toxicity of 621C47

In general, the toxic effects observed with 621C47 were similar to those reported for other phenanthridinium compounds. No calves died as a result of treatment with 621C47, but a delayed toxic effect, resembling photosensitization, was observed in one animal. This animal (Khartoum experiments) was originally infected with *T. congolense*, and the skin lesions were first noticed 57 days after subcutaneous injection with 4.0 mg./kg. of 621C47. The bull was white, with brown patches, and the lesions were found in the unpigmented areas at the base of the tail, the insides of the thighs, the back, and down the sides. The condition was typical of cases encountered in the Sudan on previous occasions, and took six months to clear. While it lasted the animal was irritable and lost condition.

Samples of serum and urine taken 91 days after the injection of the drug were analysed by Professor C. Rimington. No phylloerythrin was detected, although at this time the lesions were still severe.

At the site of injection some reaction was always present. After a dose of 2 mg./kg. swelling occurred within 3 days, sometimes becoming oedematous. After hardening, the swelling gradually disappeared, until after about 6 weeks only a slight thickening of the skin was found. The skin covering the swelling often broke down and a scar developed. Thus, of 16 calves treated with 2 mg./kg. of 621C47, 10 of them developed scars. This account describes the local reaction to the drug as seen in the Khartoum experiments when the cattle were examined weekly. The animals at Malakal were examined less frequently, but it was obvious that the reaction was much more severe here than at Khartoum.

This was probably because the Malakal cattle had to forage for food, thus dispersing the phenanthridinium solution in the subcutaneous spaces and aggravating the lesion mechanically. When the Malakal calves were examined 3 weeks after injection of the drug, every animal had a hard swelling, often measuring as much as 14 × 16 cm., and scars were observed in 56 of 104 calves. After 10 weeks a scar was found at the site of injection in almost every animal.

Two animals were given an intramuscular injection of 2.0 mg./kg. of 621C47 into the buttock and no swelling or scar developed with this route of injection.

As the toxicity of phenanthridinium compounds is revealed by the pathological picture of the liver (Goodwin and Chandler, 1952; Burdin, Plowright and Purchase, 1952), punch liver biopsies were done in animals treated with 621C47. Lack of time made it impossible to obtain a complete series from the same animals, but biopsies were made in various animals at different times after injection of the drug. Thus liver specimens were obtained from one calf 105 days after treatment with 1 mg./kg; from 4 calves 29 days after 2 mg./kg.; from 3 calves 47 days after 2 mg./kg. and 140 days after 2 mg./kg. Fat was observed in stained sections from one animal, and was present in cells lining the portal tracts. This animal had been treated with 2 mg./kg. of 621C47 and the biopsy specimen was taken 47 days after dosing. The other specimens were normal.

It was found that 150C47 gave a more severe local reaction than did 621C47; but no further observations on the effect of this drug were made.

Summary

1. Two phenanthridinium derivatives, 621C47 (2 : 7-diamino-9-α-thienyl-10-methylphenanthridinium chloride) and 150C47 (2 : 7-diamino-9-*p*-aminophenyl-10-methylphenanthridinium chloride), were given trials in the Sudan against *Trypanosoma congolense* and *T. vivax* in cattle.

2. Under laboratory conditions, a dose of 1 mg./kg. of 621C47, or of 150C47, eradicated infections of *T. congolense* in cattle; but *T. vivax* infections required 2 mg./kg.

3. Neither 621C47 nor 150C47 proved effective in non-toxic doses against an antrycide-resistant strain of *T. congolense*; nor was 621C47 effective against a dimidium-resistant strain of *T. congolense*.

4. Under field conditions in the S. Sudan, 2 mg./kg. of 621C47 eradicated *T. congolense* and *T. vivax* infections in calves.

5. 621C47 showed no prophylactic activity.

6. When given subcutaneously, 621C47 caused swelling, ulceration and scarring of the skin. A delayed toxic effect was seen in one animal after a large subcutaneous dose (4 mg./kg.). In two experiments, intramuscular injection gave no local reaction.

We would like to thank Mr. J. T. R. Evans, Director, Sudan Veterinary Service, for his help and advice throughout this work, Dr. L. P. Walls and Dr. T. Dewing, who prepared the phenanthridinium compounds, and Dr. L. G. Goodwin, who cut and prepared frozen sections of the liver biopsies. We are also grateful to Saad Mehanna, Dip. Vet. Sci. (Khartoum), for supervising the experiments at Malakal, and to Ibrahim Abdulla, head stockman at Khartoum North.

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QUINIDINE AND ANTICHOLINESTERASES ON RABBIT AURICLES

BY

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In a recent paper Burn and Kottegoda (1953) have described the action of eserine on the isolated auricles of the rabbit heart. They found that in most preparations low concentrations decreased the rate of beating, though in some they increased the rate. Higher concentrations always decreased the rate and finally arrested the contractions, and then the auricles were found to be electrically inexcitable. The effect on the amplitude of contraction was different, for low and medium concentrations increased it; high concentrations, however, reduced it. It was stated that the effect of eserine was likely to be that of an anticholinesterase, since DFP had a similar action on the rate leading to arrest of the beat, while neostigmine in high concentration had a different action, increasing the rate, neostigmine being known (e.g., from the work of Riker and Wescoe, 1946) to have an action of its own in addition to its action as an anticholinesterase.

In the following account we describe some experiments which appear to relate the action of high concentrations of eserine to the action of quinidine, and we describe also the effect of various other anticholinesterases on the auricles. We have also examined the effect of hexamethonium and of cocaine and procaine on the action of eserine.

METHODS

All observations have been made on auricles dissected from the heart of a freshly killed rabbit so as to be free from other tissue. The auricles were suspended in a bath of Locke's solution at 29° C., vigorously aerated with oxygen. The capacity of the bath was 35 ml. The Locke's solution contained in 1 litre 9 g. NaCl, 0.42 g. KCl, 0.24 g. $CaCl_2$, 0.5 g. $NaHCO_3$ and 2 g. dextrose. The contractions were recorded by a straw lever against the pull of a light spring. The natural frequency of the lever was much greater than the highest rate of contraction.

RESULTS

Action of Quinidine.—We have examined the action of quinidine in the light of the observation of Dawes (1946) that it is a substance which has been shown to reduce the action of acetylcholine (ACh) in all forms of muscle. Thus in the presence of quinidine not only the stimulant action of ACh on the frog rectus and the rabbit intestine is reduced, but also the inhibitory action on the rabbit auricles.

A small concentration of ACh (0.16×10^{-7} g./ml.) caused a slight inhibition of rabbit auricles as shown in Fig. 1. When this concentration was applied in the presence of eserine 10^{-6} g./ml., the contractions were arrested. This effect of eserine was described by Webb (1950). The contractions were resumed when the bath fluid was changed. The arrest was produced a second time by the same concentrations of eserine and ACh, and quinidine (1.5×10^{-5} g./ml.) was then added. The contractions began again. This result was con-

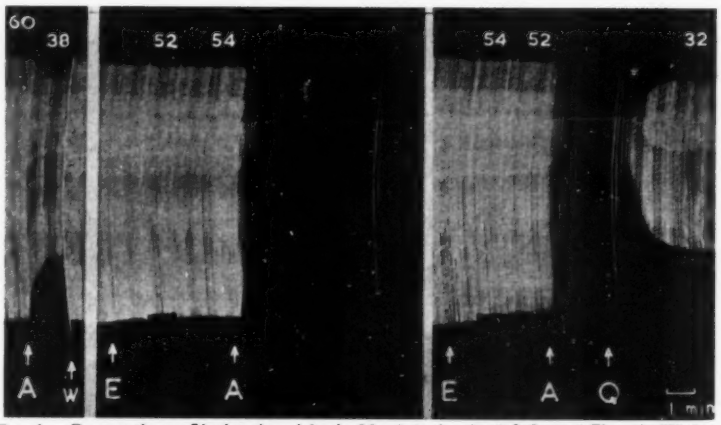

FIG. 1.—Contractions of isolated auricles in 35 ml. bath. At A 0·6 μg. ACh. At W the bath fluid was changed. At E, eserine 10^{-6} g./ml. At Q, 0·5 mg. quinidine sulphate was added. The numbers above the record are the rate per min.

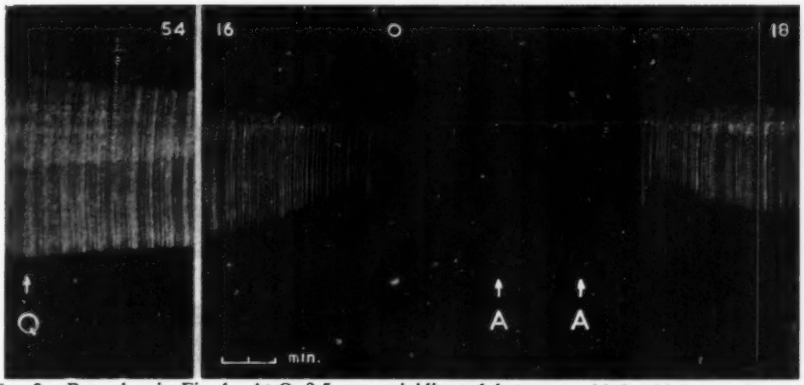

FIG. 2.—Record as in Fig. 1. At Q, 0·5 mg. quinidine sulphate was added, which caused arrest in 30 min. At A, 30 μg. ACh was added.

sistent with the view that quinidine reduced the action of ACh on the auricles so that arrest of the beat was no longer maintained.

A second experiment illustrated in Fig. 2 was carried out in which quinidine (1.5×10^{-5} g./ml.) was added to the bath containing freshly dissected auricles. When this was allowed to act, both amplitude and rate of beat were reduced, until at the end of 30 min. the contractions stopped. When ACh (2×10^{-6} g./ml.), was added the contractions began again after a latency of about 3 min. This result was obtained in 8 out of 8 trials on 6 auricles; it was consistent with the view that quinidine reduced the action of ACh endogenously produced, so that the contraction could not be initiated, or could not be conducted. When the concentration of ACh was raised by adding it to the bath the contractions began again. The amount of ACh which was added before the beat began again varied widely from 5 μg. to 2.25 mg.; the mean amount in 9 experiments was 0.42 mg., or a concentration of 1.2×10^{-5} g./ml.

A variant of this experiment, illustrated in Fig. 3, was also carried out. Quinidine was added as before and the drum was stopped while it reduced the amplitude and the rate; the rate was initially 78 per min. and fell to 48 per min. ACh (0.4×10^{-7} g./ml.) was then added; the rate briefly slowed and then the amplitude and the rate both increased, the rate to 66 per min. A further addition of ACh (1.5×10^{-7} g./ml.) was followed by inhibition and arrest of the contractions. Thus in the auricles after treatment with quinidine a stimulant action of ACh was observed in one concentration and an inhibitory action in higher concentration. This experiment was repeated three times with the same result.

The Action of Eserine.—We discovered that when the contractions of the auricles were arrested by a high concentration of eserine the addition of ACh was followed by resumption of the beat as shown in Fig. 4. In this experiment the eserine concentration was 3.4×10^{-4} g./ml. The beat was arrested in 5.5 min. ACh was then added in small amounts, and the beats began again when the concentration was 0.3×10^{-6} g./ml. After further additions of ACh the rate fell from 42 to 32, and

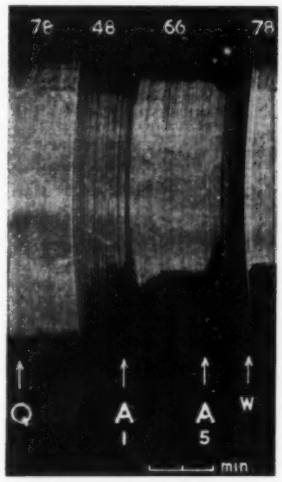

FIG. 3.—Record as before. At Q, 0·5 mg. quinidine sulphate added. The drum was stopped and started again when the rate had fallen to 48. At A_1, 1 μg. ACh was added, and the rate rose to 66. At A_5, 5 μg. ACh was added.

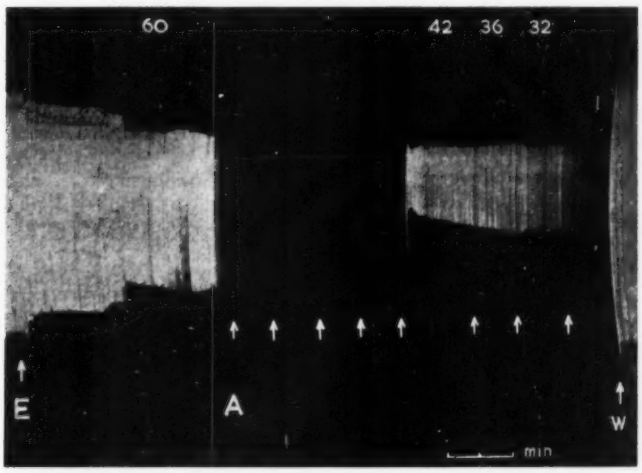

FIG. 4.—Addition of 12 mg. eserine depressed the amplitude and arrested the contractions. At the arrows, ACh was added, 0·1 μg., 0·5 μg., 1·0 μg., 3·5 μg., 5 μg. The contractions began again. Further additions were 1 μg., 5 μg. and 10 μg.

when the concentration reached 10^{-6} g./ml. the beat stopped again. Thus, as under the influence of quinidine, so under the influence of eserine it was possible to observe a stimulant action of ACh in one concentration and an inhibitory action in a higher concentration. The beat was resumed after the addition of ACh in 13 out of 14 trials in 10 auricles, but the amount of ACh used was much less than when the beat was arrested by the action of quinidine, the mean amount being only 10 μg. or a concentration of 0.3×10^{-6} g./ml.

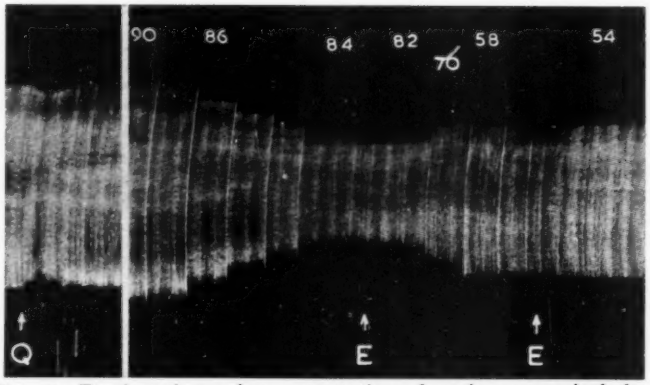

FIG. 6.—To show that a low concentration of eserine antagonized the depression of the amplitude by quinidine but accelerated the depression of the rate. Q = 0·5 mg. quinidine; E = 100 μg. eserine.

Effect of ACh in the Presence of Eserine.—The observation, that when the contractions stopped in the presence of a high concentration of eserine the addition of ACh was followed by resumption of the beat, showed that the contractions did not stop because of an accumulation of ACh. We examined the effect of ACh in the presence of different concentrations of eserine. As Fig. 1 shows, the concentration of 10^{-6} g./ml. greatly intensified the inhibitory action of ACh. A concentration of 2×10^{-4} g./ml. which slowed the rate in one experiment from 66 to 40 per min. abolished the inhibitory action of a similar amount (1 μg.) of ACh, while a concentration of 5×10^{-4} g./ml. which slowed the rate to 16 per min. reversed the action of 1 μg. ACh so that the rate was accelerated from 16 to 29 per min.

Synergism between Quinidine and Eserine.—On the assumption that eserine would cause an accumulation of acetylcholine we at first thought that eserine would antagonize the action of quinidine in causing arrest of the beat. In almost all preparations the contrary was observed and the two substances worked together. Thus in Fig. 5

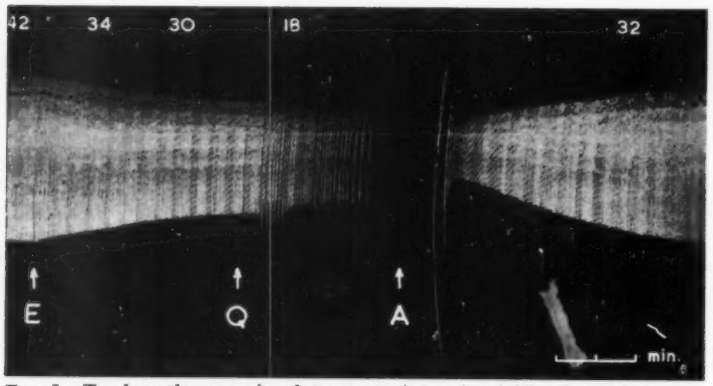

FIG. 5.—To show the synergism between eserine and quinidine in causing arrest of the beat. Eserine alone in a concentration 10^{-4} g./ml. does not arrest the beat, and 0·5 mg. quinidine alone arrests it only after 20–30 min. Together they caused arrest as shown. 100 μg. ACh then caused resumption of the beat.

eserine was added in concentration 10^{-4} g./ml. The rate fell, but the amplitude was not greatly reduced; after the addition of quinidine the beat stopped in 3 min., beginning again after the addition of ACh. In other experiments quinidine was added first; the addition of eserine in a concentration which alone would not lead to arrest of the beat shortened the time before the arrest. There were, however, experiments in which antagonism was shown. In two of these, one of which is illustrated in Fig. 6, the addition of quinidine was followed by a fall in rate and amplitude; the addition of eserine accelerated the fall in rate, but increased the amplitude.

Action of Neostigmine.—The action of neostigmine shown in Fig. 7 resembled that of eserine only in the effect of low concentrations on the rate; this slowed progressively to a mean value of 65% of the initial rate in the presence of molar concentrations 10^{-7}, 10^{-6}, and 10^{-5}. In higher concentrations the rate declined less and even accelerated above the initial rate. The amplitude was not appreciably affected by any concentration of neostigmine. Neostigmine was the only substance tested which did not cause arrest of the beat in high concentrations. Even in the presence of a concentration as high as 10^{-2}M, the rate and amplitude were not diminished. Although high concentrations of neostigmine did not arrest the contractions, they reduced the inhibitory action of ACh as shown in Fig. 8.

Action of DFP.—In the presence of molar concentrations of 10^{-6} and 10^{-5} DFP the rate decreased to 93 and 67% of the initial rate as shown in Fig. 7. In the presence of 10^{-4}M and 10^{-3}M the

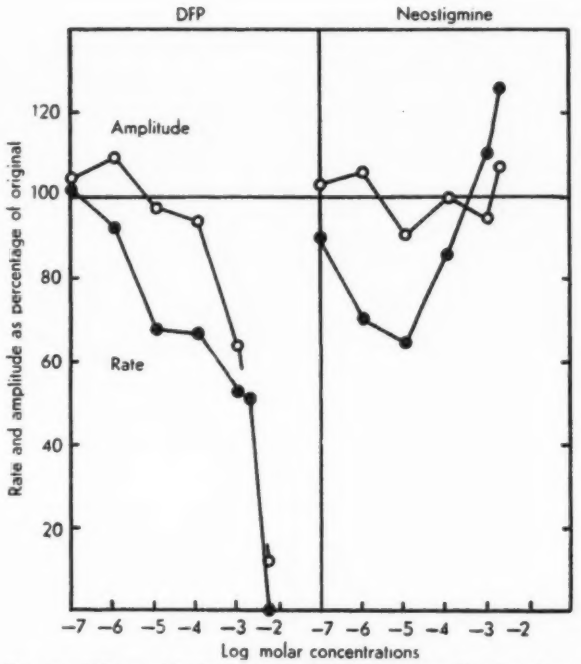

FIG. 7.—Effects of DFP and of neostigmine on amplitude and rate of rabbits' isolated auricles. Ordinates: rate and amplitude as a percentage of the initial, taken as =100. Abscissae: logarithms of the molar concentrations. Open circles, amplitude. Closed circles, rate. The effect was that recorded at the end of 30 min.

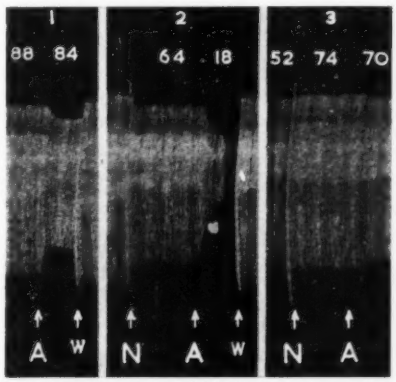

FIG. 8.—To show reduction of inhibitory action of ACh by high concentrations of neostigmine. (1) A=0·6 μg. ACh. (2) N=10^{-6}M neostigmine; A=0·6 μg. ACh. (3) N=10^{-3}M neostigmine: A=0·6 μg. ACh.

rate declined a little but not much further. In the presence of 4×10^{-3}M the contractions were arrested. The amplitude was little affected in the presence of concentrations up to 10^{-4}M, but above this the amplitude was greatly reduced, so that it fell to zero with the rate.

Action of Other Anticholinesterases.—The mean results with three other anticholinesterases are given in Table I. Results with Nu 683 (the dimethyl carbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide) were of interest because this

TABLE I

ACTION OF ANTICHOLINESTERASES ON RATE AND AMPLITUDE OF RABBITS' ISOLATED AURICLES

Effects are measured as percentage of initial value after 30 min.

Molar Concn.	Nu 683		Nu 1250		BW 284C51	
	Rate	Amplitude	Rate	Amplitude	Rate	Amplitude
10^{-7}	98·5	103	75	104	86	100
10^{-6}	65·5	111·5	80	112	86	98
10^{-5}	69	111	135	126	96	98
10^{-4}	75	109	157	112	103	99
10^{-3}	74·5	58·5	143	84	101	81

substance was shown by Hawkins and Gunter (1946) to be a highly specific inhibitor of pseudocholinesterase, while results with BW 284C51 (the dimethobromide of 1 : 5-di(*p*-N-allyl-N-methylaminophenyl)-pentan-3-one) were interesting because this substance was shown by Austin and Berry (1953) to be a specific inhibitor of true cholinesterase. Nu 1250 (the N-*p*-chlorophenyl-N-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide) was found to be a rather less specific inhibitor of true cholinesterase (Hawkins and Mendel, 1949). Each inhibitor was tested on 4 or 5 auricle preparations.

All three inhibitors failed to affect the amplitude in concentrations less than 10^{-3}M, and in this concentration Nu 683 depressed the amplitude more than did the others. At a concentration of 10^{-6}M the rate was slowed to 66% in the presence of Nu 683, a lower figure than was observed with the other two inhibitors. At concentrations of 10^{-5}M, 10^{-4}M, and 10^{-3}M there was no further fall in rate in the presence of Nu 683, while in the presence of Nu 1250 the rate was accelerated above the initial value, and in the presence of BW 284C51 the rate returned to the initial value. At a concentration of 2×10^{-3}M, 3 out of 5 auricles were arrested in the presence of Nu 683, and 1 out of 4 in the presence of Nu 1250. In the presence of BW 284C51 auricles were arrested only in concentrations as high as 8×10^{-3}M and 10^{-2}M.

In the presence of concentrations 10^{-4}M and 10^{-3}M, the inhibitory action of ACh on the auricles was reduced by all three anticholinesterases.

Observations with Hexamethonium.—In the auricles endogenous ACh may be liberated from nerve endings originating in ganglia or may be formed in non-nervous tissue. The action of nicotine on the auricles which is partly inhibitory and partly stimulant is abolished by hexamethonium (Kottegoda, 1953). Some observations were therefore made to see whether the action of eserine in slowing or arresting the contractions, or of neostigmine and Nu 1250 in increasing the rate of contractions,

was modified in the presence of hexamethonium. The results are given in Table II. This shows that hexamethonium did not modify the slowing of the rate caused by eserine (10^{-5} g./ml.). Further, hexamethonium did not affect the arrest of the auricles which occurred in the presence of a high concentration of eserine (4×10^{-4} g./ml.); nor the resumption of the beat which followed the addition of ACh. The acceleration of the rate by neostigmine (10^{-5}M) and by Nu 1250 (10^{-4}M) was also unaffected by the presence of hexamethonium.

TABLE II

EFFECT OF HEXAMETHONIUM ON ACTION OF ANTICHOLINESTERASES ON RATE OF AURICLES

The effect is expressed as the percentage of the initial value

Substance and Concn.	Concn. Hexamethonium	No. of Auricles	No. of Observations	Mean Effect on Rate
Eserine 10^{-5} g./ml. ..	—	4	6	73%
" " ..	3×10^{-4} g./ml.	4	6	76%
Eserine 4×10^{-4} g./ml.	—	2	5	Arrest
" "	3×10^{-4} g./ml.	2	5	Arrest
Neostigmine 10^{-5}M ..	—	4	6	143%
" " ..	10^{-4} g./ml.	4	6	148%
Nu 1250 10^{-4} ..	—	2	2	116%
" ..	10^{-4} g./ml.	2	2	121%

Hexamethonium did not modify the rate of auricles which had not been exposed to the action of other substances; this observation was made in 3 preparations using a concentration of 3×10^{-4} g./ml. Hexamethonium in this concentration, however, raised the rate of auricles previously exposed to eserine. It was rarely possible to remove all eserine by repeated change of the bath fluid, and the residual slowing was antagonized by hexamethonium in 9 auricles, the mean rise in rate being 20%. We believe this to be due to a very weak atropine-like action which hexamethonium possesses, an action too weak to modify the effect of ACh either on the auricles or in the heart-lung preparation (as observations recently made here by one of us have shown), but perhaps explaining the inhibition of the action of ACh in the isolated and perfused cat heart described by Perry and Talesnik (1953). This action, one thousand times weaker than that of atropine, can, however, be seen to lessen the action of eserine in the heart-lung preparation.

Observations on Cocaine and Procaine.—Cocaine hydrochloride in a concentration of 5×10^{-6} g./ml. abolishes the peristaltic reflex in the isolated guinea-pig intestine, and we found that it abolished the action of nicotine on the auricles. We therefore used this concentration in the observations on eserine. By itself cocaine produced some increase of the rate in occasional preparations, but commonly a decrease. This made it difficult to determine the effect of cocaine on the action of eserine. In one experiment in which eserine 10^{-6} g./ml. was used, a concentration likely to exert an anticholinesterase action only, eserine applied for 20 min. reduced the rate to 70% of the initial value, eserine and cocaine together reduced it to 76%, and cocaine alone to 88%. The initial rates were almost the same in each case. Thus cocaine may have reduced the effect of eserine, but did not abolish it. Other experiments gave similar inconclusive results.

We observed that higher concentrations of cocaine (4×10^{-5} g./ml.) and of procaine led to arrest of the beat, but the addition of ACh was not followed by resumption of beating.

DISCUSSION

The results obtained may be considered in the light of the hypothesis that some of the effects of quinidine on cardiac muscle are due to its property of diminishing the action of ACh. This diminution, we may suppose, would be due to quinidine competing for receptors on which ACh acts. When the auricles are arrested by ACh in the presence of a low concentration of eserine, the addition of quinidine to the bath causes the beats to start again. This can clearly be explained as a diminution of the action of ACh, which is no longer able to maintain the state of arrest.

When quinidine is added to the bath containing auricles recently set up the rate and amplitude decline and the beat stops. This action can only be due to a diminution of the action of ACh if the rate and force of the beat depend on the local formation of ACh, as Bülbring and Burn (1949) suggested. The fact that the auricles arrested by quinidine begin to beat again when ACh is added to the bath is in accordance with the suggestion. We may therefore suppose that quinidine arrests the auricles by occupying more and more of the receptors on which the intrinsic ACh acts, until the beat stops. When more ACh is added, some of the quinidine molecules are dislodged from the receptors, and the beat begins again.

Further support for the view that quinidine causes arrest of the beat by diminishing the action of intrinsic ACh comes from the observations with eserine. If quinidine occupies receptors on which ACh acts, other substances which combine with these receptors should also arrest the beat. Now eserine combines with cholinesterase, and ACh combines with cholinesterase, so it is reasonable

to expect that eserine would have some affinity for receptors on which ACh acts. Our results show that high concentrations of eserine arrest the beat just as quinidine does, and that the addition of ACh then starts the beat again. In causing arrest of the beat eserine and quinidine were found to be additive in their action.

Arrest of the beat by eserine might be thought to be caused by accumulation of ACh. Experiments by one of us (S.B.) with U. Trendelenburg showed that the amount of ACh present in auricles stopped by eserine was not greater than that found in normal auricles. Moreover, the resumption of the beat when ACh was added proves that the arrest was not due to excess. Further, neostigmine did not act like eserine; the highest concentrations of neostigmine did not arrest the beat.

Arrest of the beat by eserine is perhaps related to a failure of conduction for the following reasons. The effect of eserine is like that of quinidine, and quinidine has long been known to depress conduction. Rothschuh and Bammer (1952) have observed that eserine diminishes the rate of conduction in a strip of frog ventricle, while neostigmine increases it. As already mentioned, neostigmine did not cause arrest of the auricles. Auricles arrested by eserine were found by Burn and Kottegoda (1953) to be electrically inexcitable. The resumption of the beat after the addition of ACh may be explained by a restoration of conduction. Prinzmetal, Corday, Brill, Oblath, and Kruger (1952) showed that infusion of ACh into the dog prevents the failure of conduction which otherwise occurs when the heart is driven electrically at very high rates. Their records show that the conduction time shortens under the influence of ACh. Burgen and Terroux (1953) state, on the other hand, that in isolated cat auricles ACh slows the rate of conduction, basing their conclusions on the action of carbachol. Our colleague, Dr. E. M. Vaughan Williams, has observed that the rate of conduction in rabbit auricles is quickened by ACh. It is probable that both effects can be obtained. Whether the arrest of the beat by quinidine and by eserine is in fact due to a failure of conduction must be determined by other methods.

Before leaving the discussion of the action of quinidine, reference should be made to a view put forward by one of us (Burn, 1953) that there is an optimal concentration of ACh for cardiac activity and that amounts less than this or more than this cause diminished activity. This hypothesis suggests that the usual inhibitory action of ACh is an effect of excess. The hypothesis may be illustrated by reference to Figs. 3 and 4, where both the stimulant and the inhibitory action of ACh is seen, the first in low concentration and the second in higher concentration. This interpretation, however, ignores what is shown in Fig. 3 and what was seen in several experiments, that the low dose of ACh produced a further slowing of the rate as its immediate effect, this phase being transient and giving place to increased rate and amplitude. That is to say, the ordinary inhibitory action of ACh was detectable as a preliminary to the stimulant action, and therefore the inhibitory action on the rate cannot be considered as the effect of excess.

Burgen and Terroux (1953) have found that the resting potential of the cat auricle is increased by ACh, a result which agrees with the finding of Gaskell (1887) that the demarcation potential of the auricle of the turtle was increased by vagus stimulation. These observations suggest that the inhibitory action of externally applied ACh is due to hyperpolarization, and they do not fit with a conception that added ACh has both a stimulant and an inhibitory action on the rate. Eserine, on the other hand, in low concentration can accelerate the rate of the isolated auricles, though it usually retards it; similarly it can accelerate the rate of the perfused frog heart. Thus it would appear that changes in the concentration of endogenous ACh due to inhibition of cholinesterase may modify the rate in either direction.

The idea that ACh may stimulate in low concentration and inhibit in high concentration may, however, apply to its action on conduction and also on the force of the beat. In some of our experiments a low concentration of eserine was found to antagonize the depressant action of quinidine on the amplitude of contractions, though collaborating with it in reducing the rate. This would suggest that ACh may have a double action on the force of the beat.

The gradual decline in the rate and amplitude of the contractions of auricles exposed to proguanil until the beat is arrested (Burn and Vane, 1949) is probably similar to the action of quinidine, since in both cases the beat is resumed on addition of ACh. We found that cocaine, procaine, and atropine, which Dawes (1946) showed to act like quinidine on the electrically driven auricles, also had a similar action on those beating spontaneously, though when arrest was caused by these substances the addition of ACh was not followed by resumption of the beat.

The effect of other anticholinesterases, including neostigmine, was similar to eserine in one respect only, that low concentrations, 10^{-7}M and 10^{-6}M (or, for DFP, 10^{-6}M and 10^{-5}M) decreased the rate.

We think it likely that this action was an uncomplicated anticholinesterase action and indicated that the contracting auricles were forming ACh and that this formation was controlling the rate. We attempted to discover whether the formation took place in ganglion cells by testing the effect of hexamethonium and cocaine. Hexamethonium did not modify the action of eserine (10^{-5} g./ml.). Cocaine did not appear to modify the effect of eserine (10^{-6} g./ml. or 10^{-5} g./ml.). It was, however, more difficult to be certain of this, since cocaine alone caused some slowing of the rate. On the whole, the evidence was not in favour of a nervous origin for the ACh.

If the beat is initiated by the formation of ACh at the pacemaker, the generally accepted myogenic origin of the heart beat put forward by Gaskell and Engelmann long ago would indicate a myogenic origin for the ACh.

Summary

1. Quinidine, which diminishes the action of ACh in all forms of muscle, causes the rate and amplitude of the beat of rabbits' isolated auricles to diminish until the contractions cease. If ACh is added to the bath, the contractions are resumed. A further addition of ACh may inhibit the contractions.

2. If eserine is added to the bath containing contracting auricles, when the concentration reaches a point between 10^{-4} and 10^{-3} g./ml. the contractions cease. If ACh is added to the bath, the contractions are resumed. A further addition of ACh inhibits the contractions.

3. Quinidine and eserine appear to act as synergists in causing arrest of the auricles.

4. Low concentrations of other anticholinesterases, neostigmine, DFP, Nu 683, Nu 1250, BW 284C51, act like eserine in slowing the rate.

5. Higher concentrations of neostigmine increase the rate, and do not cause arrest of the beat; neostigmine does not modify the amplitude.

6. DFP depresses both rate and amplitude in high concentrations and causes arrest.

7. Reasons are given for regarding the action of quinidine and of high concentrations of eserine in arresting the auricles as perhaps due to an effect on conduction which is reversed by ACh.

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A SEA ANEMONE EXTRACT (THALASSINE) WHICH LIBERATES HISTAMINE AND A SLOW CONTRACTING SUBSTANCE

BY

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The discovery of anaphylaxis was made during a study of the toxicity of extracts of the stinging tentacles of various coelenterates such as *Physalia* and *Actinia* (Portier and Richet, 1902; Richet, Perret, and Portier, 1902; Richet, 1905). From a crude extract of *Actinia* tentacles these workers separated three pharmacologically distinct fractions which on intravenous injection into dogs produced severe generalized pruritus, pulmonary oedema, or sedation. They termed these fractions thalassine, congestine, and hypnotoxin respectively. Thalassine, the pruritogenic fraction, is obtained as a stable, dry powder by repeated alcohol extraction.

Since generalized pruritus occurs in dogs after the systemic administration of various drugs known to release histamine (Paton and Schachter, 1951), and since cutaneous reactions may be produced in man by the local application of anemone tentacles (Gosse, 1860; Stephenson, 1935), it seemed to us that thalassine might cause the release of histamine.

The present experiments demonstrate that thalassine does in fact release histamine from the isolated, perfused cat skin. On intravenous injection into cats it produces a prolonged increase in the plasma histamine concentration, and also liberates a substance producing a delayed, slow contraction of the guinea-pig ileum.

METHODS

Cats were anaesthetized with chloralose (80 mg./kg.) intravenously, preceded by ethyl chloride–ether induction. Blood samples taken from the femoral artery were heparinized, centrifuged, and the plasma tested for histamine on the isolated guinea-pig ileum. Blood pressure was recorded from the carotid artery by a mercury manometer.

Cat skin preparations were prepared by the method of Feldberg and Paton (1951) as modified by Feldberg and Schachter (1952). Injections were made into the saphenous artery in 0.5-ml. volumes, and the perfusate collected from the corresponding vein. The infusion was arrested for 10–20 sec. during the injection, thus permitting the injected material to remain in contact with the tissues for this period.

Histamine assay of perfusates and plasmas was carried out on the isolated guinea-pig ileum suspended in magnesium-free Tyrode solution in an 18-ml. bath. Atropine (0.2 μg.) was added to the bath after each test to abolish the spontaneous activity of the preparation. Mepyramine (0.5 μg.) was added when required to antagonize the histamine activity of the test samples.

Preparation of Thalassine from Anemone Tentacles.—Thalassine was prepared from anemone tentacles by repeated alcohol extractions, as described by Richet (1905). Sea anemones (mostly *A. equina*, but including a small proportion of *A. sulcata*) were obtained from the south coast of England and kept alive in sea water until the chemical extraction was begun. From approximately 700 g. of these tentacles, 2.85 g. of dry material was obtained. The powder was readily soluble in distilled water in amounts up to 100 mg./ml. A 1.0% (w/v) solution in saline was slightly acidic (*p*H 6.5) because acetic acid was used at one stage of the extraction to facilitate precipitation of the active material.

Two thalassine preparations were made from separate batches of sea anemones. One was more effective as a histamine liberator and was used in most experiments. Neither preparation had any effect on the guinea-pig ileum, even in high concentrations, and contained less than 0.5 μg. histamine per gram of dried powder. Fresh tentacle itself also contained only small amounts (2.0–6.5 μg./g.) of histamine on extraction with hydrochloric acid.

RESULTS

Release of Histamine From Perfused Cat Skin by Thalassine

Thalassine (0.05–5.0 mg.) was injected intra-arterially into perfused cat skin preparations in six

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TABLE I

RELEASE OF HISTAMINE FROM PERFUSED CAT SKIN FOLLOWING THE INTRA-ARTERIAL INJECTION OF SEA ANEMONE TENTACLE EXTRACT (THALASSINE)

	Amount of Thalassine Injected (mg.)	Total Histamine Collected in Perfusate (μg.)
Less potent preparation	5·0	35·0
	2·0	37·3
	0·25	0·9
More potent preparation	0·5	31·7
	0·1	16·8
	0·05	3·1

experiments. Histamine was released in every instance. The total amount collected in the perfusate in each experiment is shown in Table I. The minimal dose of thalassine required to release histamine from the isolated skin was 0.05 mg. The corresponding threshold dose of compound 48/80 was 0.001 mg. After the intra-arterial injection of thalassine, as of 48/80, the histamine concentration in the effluent was greatest in the first 10-minute sample in five of the six experiments. In one experiment, however, the release was more protracted and the histamine concentration was highest in the second 10-minute sample. The perfusates remained clear throughout, and their activity on the guinea-pig ileum was completely abolished by mepyramine.

Release of Histamine by Intravenous Injection of Thalassine in the Intact Cat

Thalassine (10 mg./kg.) was injected intravenously in six cats after cervical section of the vagus nerves. After the injection all animals showed a prolonged fall of the arterial blood pressure, with only partial recovery in one hour. The fall of blood pressure was regularly delayed for approximately 30 seconds following the injection. This delay is characteristic of substances whose main circulatory depressor action is due to the release of histamine (MacIntosh and Paton, 1949). In two experiments, thalassine also caused a slight, short-lasting, immediate fall of the blood pressure which recovered before the onset of the severe prolonged depression. Fig. 1 shows the prolonged circulatory response to thalassine, together with the short-lasting immediate fall that sometimes precedes it.

Blood samples for plasma histamine assay were taken from the femoral artery before, and at intervals of 4 to 90 minutes after, the intravenous injection of thalassine. Injection of thalassine always produced a considerable increase in plasma histamine concentration which was still detectable one hour later. The results of these experiments are shown in Table II. The histamine releasing effect of thalassine solutions was unaffected by heating for five minutes in a boiling water bath.

TABLE II

PLASMA HISTAMINE CONCENTRATIONS (μG./ML.) BEFORE AND AT VARIOUS INTERVALS AFTER THE INTRAVENOUS INJECTION OF THALASSINE (10 MG./KG.) INTO CATS

Exp. No.	Before Injection	Minutes after Injection 4	10	40	60	90
1	0·02	0·10	0·14	—	0·05	—
2	0·03	0·06	0·20	0·18	0·17	0·10
3	0·04	0·10	0·15	0·18	0·17	0·06
4	0·03	—	0·18	0·30	0·25	—
5	0·01	0·08	0·14	0·12	—	—
*6	0·02	0·11	0·21	0·28	0·17	0·12

* Solution heated in boiling water bath for 5 minutes before injection

In assaying the post-injection plasma samples for histamine, it became apparent that the later samples produced a delayed contraction of the guinea-pig ileum. However, by using small amounts of plasma for histamine assay, 0.2 ml. or less, it was possible to eliminate this effect. The quantitative nature of the plasma histamine assay was subsequently confirmed by the complete abolition of the effect of 0.2 ml. of plasma on the

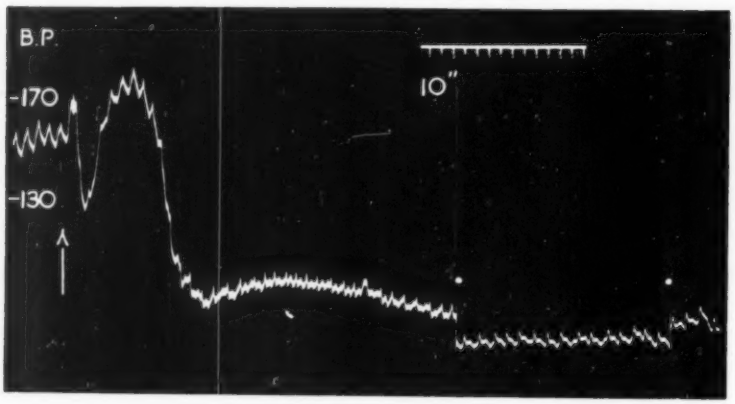

FIG. 1.—Prolonged depression of arterial blood pressure of a cat following intravenous injection of thalassine. Cat 3.0 kg., vagi cut in neck. Thalassine, 10 mg./kg., injected at arrow. Recording stopped for 15 minute intervals at dots.

guinea-pig ileum by mepyramine. The delayed contraction was itself subsequently studied.

Effect of Plasma, Obtained After Injection of Thalassine, in Producing a Delayed, Slow Contraction of the Guinea-pig Ileum

The effect of cat plasma taken before and at intervals after the intravenous injection of thalassine (10 mg./kg.) was tested on the guinea-pig ileum in the presence of mepyramine. The addition to the bath of 1.0 ml. of plasma obtained 30 minutes after injection of thalassine produced a marked contraction of the guinea-pig ileum, whereas control plasma was either without effect or produced a much smaller contraction. Such an effect was regularly observed in five experiments and the results of two of these are shown in Fig. 2. This smooth muscle stimulating action of

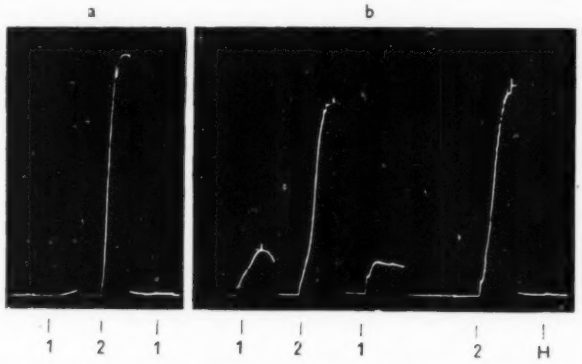

FIG. 2.—Effect of cat blood plasma taken before and 30 minutes after the intravenous injection of thalassine (10 mg./kg.) on the isolated guinea-pig ileum in the presence of atropine (0·2 μg.) and mepyramine (0·5 μg.). a, and b, are results of separate experiments. The following were added to the 18 ml. bath :— 1, 1.0 ml. cat plasma taken before injection of thalassine. 2, 1.0 ml. plasma obtained 30 minutes after injection. H, 0.3 μg. histamine.

plasma differs from that of histamine, acetylcholine, 5-hydroxytryptamine, and other smooth muscle stimulants, in that there is a delay of 10–20 seconds before contraction begins and that a longer interval, usually about 60 seconds, is required for the contraction to reach its maximum. This property of cat plasma was regularly present 30 minutes after the injection of thalassine and gradually disappeared, becoming undetectable in 90 minutes.

In one experiment, plasma obtained 30 minutes after injection of thalassine produced a greater contraction of the rat colon than did control plasma. Since this effect persisted after tryptamine desensitization it could not be caused by 5-hydroxytryptamine ; it would thus appear to be due to the same substance as produces the delayed, slow contraction of the guinea-pig ileum.

DISCUSSION

The demonstration that an alcoholic extract of sea anemone tentacles is an effective histamine releaser suggests that the pruritogenic activity of anemone extracts is due to this property. It is possible that histamine releasing substances may be widespread in nature and may be present in secretions of various organisms, such as jellyfish, lice, mites, cercaria, etc., which provoke local pruritus on contact with human skin. The potency of our thalassine preparation, which is not chemically homogeneous, is approximately 1/50th that of compound 48/80, one of the most potent histamine releasing substances hitherto studied. If the histamine releasing property of thalassine derives from a single substance, this would appear to be of the same order of potency as 48/80. The histamine releasing property, however, represents only one of the pharmacological effects of anemone tentacles. Thus, even large doses of thalassine failed to produce pulmonary oedema, whereas severe pulmonary exudation followed the injection of small amounts of a saline extract of tentacles.

The ability to cause the appearance in plasma of a substance producing a delayed, slow contraction of the guinea-pig ileum is not confined to thalassine. For example, Feldberg, Holden, and Kellaway (1938) demonstrated a " slow reacting substance " in lung perfusates following perfusion with snake venoms ; Beraldo (1950) reported the release of " bradykinin " during anaphylactic shock in dogs ; and Paton (1951) has recently described a similar activity in cat plasma after injection of 48/80. Since thalassine, snake venoms, antigen and 48/80 all release histamine, it seems that the appearance of a slow reacting substance is closely associated with the process of histamine release, as previously suggested by Paton (1951). However, although we regularly detected this slow reacting activity in cat plasma following injection of thalassine into the whole animal, it was never detected in perfusates from cat skin preparations after intra-arterial injection of thalassine—despite the release of histamine. It would thus seem that histamine can be released from tissues without the release or formation of a slow contracting substance. It is also probable that there exists a variety of substances able to provoke a slow contraction of the guinea-pig ileum.

SUMMARY

1. Thalassine, an alcohol extract of sea anemone tentacles, has been shown to possess potent histamine releasing activity.

2. It regularly released histamine from the perfused cat skin, and raised the plasma histamine concentration in this species following its intravenous administration.

3. On intravenous injection in the cat, thalassine also caused the appearance in plasma of a substance producing a delayed, slow contraction of the guinea-pig ileum.

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THE PRESENCE OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND A POTENT, SLOW CONTRACTING SUBSTANCE IN WASP VENOM

BY

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The demonstration that an alcoholic extract of sea anemone tentacles contains a potent histamine liberator (Jaques and Schachter, 1954) prompted us to test the possibility that wasp venom might also possess such activity. Saline extracts of wasp venom were accordingly studied. We found that only some wasp glands contained venom capable of releasing histamine from perfused cat skin preparations. The venom itself, however, regularly contained large amounts of histamine and also an extremely potent, unidentified constituent which produced a delayed, slow contraction of the guinea-pig ileum. This latter activity resembled that which we detected in cat plasma following intravenous injection of the histamine releasing fraction of anemone extract.

5-Hydroxytryptamine could not be detected in crude venom by the guinea-pig ileum because of the presence of two other potent smooth muscle stimulants. It was, however, identified as a constituent of the venom by paper chromatographic analysis; its presence in the eluate was subsequently confirmed pharmacologically by its action on the guinea-pig ileum. Histamine was also identified by these procedures.

We are greatly indebted to Dr. T. S. Work for carrying out the chromatographic analyses.

METHODS

The histamine assay and the perfusion of cat skin preparations were performed as described in the preceding paper (Jaques and Schachter, 1954). Histamine was used as acid phosphate, 5-hydroxytryptamine as creatinine sulphate, tryptamine as hydrochloride, atropine as sulphate, and mepyramine as maleate. Values for histamine and 5-hydroxytryptamine are expressed as base throughout.

Isolated rat colon preparations for testing the smooth muscle stimulating action of wasp venom were suspended in modified Locke solution, containing half the usual amount of glucose and a quarter of the usual amount of calcium; this solution reduces spontaneous activity of the isolated muscle (Gaddum, Peart, and Vogt, 1949). As reported by Dalgliesh, Toh, and Work (1953), this preparation was insensitive to 100 μg. histamine added to the bath, thus enabling us to study the non-histamine smooth muscle action of wasp venom without having to antagonize the action of histamine present in the venom.

Wasp Venom.—The common wasp (*Vespa vulgaris*) was used as a source of venom. The wasp was first immobilized in the cold, then pinned on its back and the ventral part of the three last abdominal segments cut away. The sting was then grasped with fine forceps and the entire venom apparatus, consisting of the venom sac and glands plus Dufour's alkaline gland, was gently lifted out of its bed by pulling on the sting. Care was taken to free the venom apparatus from other parts, such as fat body, or rectum, which sometimes adhered to it. The material so obtained from a large number of wasps was dried *in vacuo* over P_2O_5 and stored at 0° C. for future testing. The percentage of soluble venom in the dried venom apparatus was determined by weighing 100 such structures and subsequently grinding them in distilled water. The insoluble residue was dried *in vacuo* over P_2O_5 for 48 hours and weighed. It was thus possible to calculate the percentage of soluble material in the venom apparatus, and in two such analyses 27.0 and 30.4% respectively of the dry weight of the structure was soluble. For convenience, we therefore considered 30% of the dry weight of the apparatus to consist of soluble venom; throughout the investigation expressed weights of venom refer to 30% of the weight of the entire dry venom apparatus. The mean weight of a single dry apparatus was 0.63 mg.

In other instances these structures were dissected out, weighed fresh, dissolved in saline and the supernatant fluid tested.

Chromatography of Wasp Venom.—Dried venom apparatus weighing 115 mg.—and consisting of approximately 35 mg. venom—was dissolved in distilled

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water, and 5 mg. venom was used for chromatography on Whatman No. 3 paper, 18 × 22 in. The venom solution was spread along a line 10 cm. from one edge of the paper and approximately 30 cm. in length, and markers (25.0 μg. histamine and 25.0 μg. 5-hydroxytryptamine) were placed on spots outside the main line of venom. The sheet was irrigated overnight with the butanol–acetic acid mixture of Campbell, Work, and Mellanby (1951), dried and cut into five strips, the two outer strips containing the marker samples of 5-hydroxytryptamine and histamine and the other three strips (two narrow and one wide) containing the venom. Ehrlich's reagent (Dalgliesh, 1952) was sprayed on one of the marker strips and on one of the narrow venom strips; 0.1% ninhydrin was sprayed on the other marker strip and on the second narrow venom strip.

Results

Pharmacological Identification of Histamine in Wasp Venom

The venom sac and glands only, of 15 wasps, were dissected out; each was separately weighed, ground in a glass tissue grinder in a measured quantity of saline and the venom solution assayed for histamine. It was evident that venom produced a contraction of the guinea-pig ileum only partially due to histamine since the contraction was unusually protracted and the ileum failed to relax on washing out the bath as readily as it does after histamine. This was substantiated by the demonstration that wasp venom caused a delayed, slow contraction of the guinea-pig ileum in the presence of mepyramine. However, by assaying maximal dilutions of wasp venom which still elicited an immediate contraction of the guinea-pig ileum, it was possible to assay activity which could be abolished by mepyramine. It was possible in this way to assay the histamine content of untreated venom. The results of the 15 assays are shown in Table I. The mean histamine concentration was 4.3 mg./g. fresh venom sac plus its glands.

In view of the possible influence of the slow contracting effect of untreated wasp venom on the assay of its histamine content, the histamine concentration was also determined by Code's (1937) modification of the method of Barsoum and Gaddum, which involves prior removal of protein with trichloroacetic acid and subsequent boiling of the filtrate in concentrated hydrochloric acid. This treatment removed all traces of the venom material producing the delayed slow contraction of the ileum; the histamine assay then presented no difficulties. This analysis was performed on a pool of 160 venom apparatuses which had been dried *in vacuo* over phosphorus pentoxide and stored at 0° C. The mean histamine concentration by this method was 4.8 mg./g. of dry venom apparatus. Since only approximately 30% of the dry apparatus consists of soluble material, the mean concentration of histamine in the dry venom itself is of the order of 16 mg./g. This, to our knowledge, is a far higher histamine concentration than hitherto reported in animal tissues or secretions.

TABLE I

HISTAMINE CONTENT OF SINGLE WASP VENOM SACS PLUS THEIR GLANDS (MG./G. WET WEIGHT)

Weight of Venom Sac + Glands (mg.)	Histamine Content (Total; μg.)	Histamine Content (mg./g.)
0·6	3·6	6·0
0·6	1·8	3·0
0·5	1·5	3·0
0·7	0·6	0·9
1·1	3·0	2·7
0·9	2·4	2·7
0·8	1·6	2·0
0·8	2·5	3·1
0·7	5·0	7·1
0·7	8·5	12·1
0·7	6·2	8·9
1·0	1·9	1·9
0·9	2·5	2·8
0·8	2·8	3·5
0·7	3·4	4·9
Mean 0·8	3·2	4·3

Unidentified Substance in Venom Producing a Delayed, Slow Contraction of the Guinea-pig Ileum

Wasp venom regularly produced a characteristic delayed, slow contraction of the guinea-pig ileum in the presence of atropine and mepyramine; it also persisted after tryptamine desensitization (Gaddum, 1953). There was a delay of approximately 10 seconds before contraction began, and contraction reached its maximum in 45–60 seconds. Subsequent relaxation on washing out was somewhat more prolonged than after a histamine contraction. These facts readily eliminate the possibility that this contraction is due to known smooth muscle stimulants such as acetylcholine, histamine or 5-hydroxytryptamine. Marked contractions were obtained with 5.0 μg. of venom and as little as 0.25 μg. could be detected. Since the bath volume was about 18 ml., venom is highly effective in producing this contraction in concentrations of 1 in 3 million, or less. The effect was reproducible—though not so precisely as with histamine—and graded contractions were obtained. It was necessary, however, to lengthen the interval between contractions to about four minutes, otherwise some refractoriness developed. Fig. 1 shows the effect of graded doses of venom (0.25–5.0 μg.) on the guinea-pig ileum in the presence of

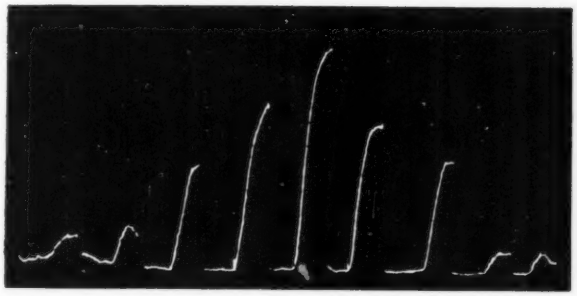

FIG. 1.—Effect of graded doses of wasp venom on the isolated guinea-pig ileum in an 18 ml. bath in the presence of atropine (0.2 μg.) and mepyramine (0·5 μg.). Contraction time, 50 seconds. Numbers 1 to 5 indicate contractions resulting from the addition to the bath of 0·25, 0·5, 1·25, 2·5 and 5·0 μg. wasp venom, respectively.

atropine and mepyramine. Fig. 2 shows the effect on a more sensitive ileum preparation of 5.0 μg. venom following tryptamine desensitization, in addition to the presence of atropine and mepyramine. Tryptamine desensitization greatly reduced the effect of 5-hydroxytryptamine whereas that of venom was unaffected. The delay in onset and the subsequent character of the contraction were similar to the slow contraction produced by cat plasma after intravenous injection of thalassine (Jaques and Schachter, 1954). The constituent of venom producing this contraction was little affected by heating venom at neutral *p*H in a boiling water bath for 5 minutes, but was destroyed after a few minutes of this procedure in 0.1N-NaOH. It was also completely eliminated by Code's method of histamine extraction. The venom regularly contracted the isolated rat colon though the sensitivity of this preparation was less than that of the guinea-pig ileum. Its action on this organ was little if at all affected by tryptamine desensitization indicating that this effect was not due to 5-hydroxytryptamine.

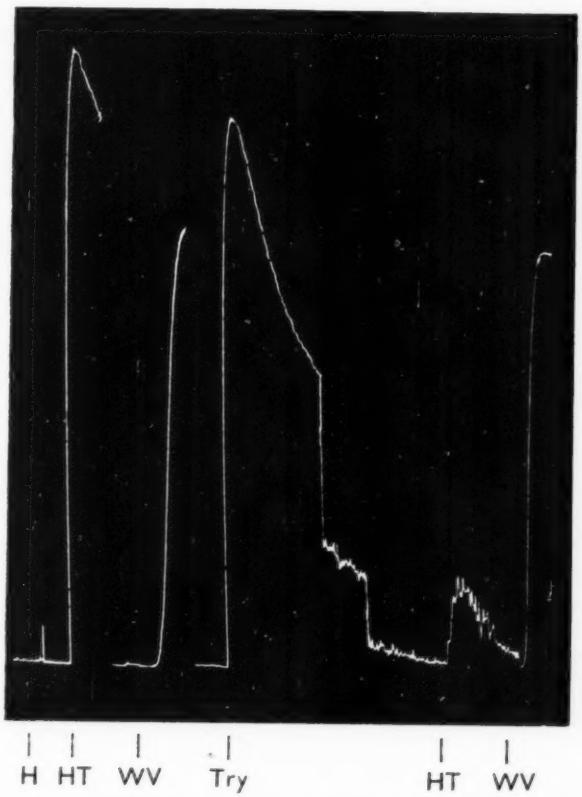

FIG. 2.—Effect of wasp venom on the isolated guinea-pig ileum after tryptamine desensitization. Atropine (0 2 μg.) and mepyramine (0·5 μg.) present in bath throughout. Continuous tracing indicates that solutions were not washed out between tests. Contraction time, 50 seconds. H, 1·0 μg. histamine; HT, 2·0 μg. 5-hydroxytryptamine; WV, 5·0 μg. wasp venom; Try, 200.0 μg. tryptamine.

Identification of Histamine and 5-Hydroxytryptamine in Wasp Venom by Paper Chromatography

The marker strip (see "Methods"), sprayed with Ehrlich's reagent (dimethylaminobenzaldehyde in hydrochloric acid) and dried in air, developed the single grey-blue spot characteristic of 5-hydroxytryptamine. The narrow strip from the chromatographed venom also developed a similar spot of the same R_f value and the same colour. No other coloured component was seen on this strip (for R_f values of 5-hydroxytryptamine and related compounds see Dalgliesh, Toh, and Work, 1953).

The marker strip sprayed with ninhydrin showed a single spot, close to the origin, corresponding to the known colour and R^f of histamine. The venom strip sprayed with ninhydrin showed a complex series of purple bands. The unsprayed paper containing the main bulk of the venom was cut into strips parallel to the origin and numbered from 1 to 5 so that, as far as possible, each strip corresponded to a single colour zone on the sprayed strips. Each strip was eluted with a mixture of 50% ethanol-water, the eluates taken to dryness and each redissolved in approximately 1 ml. of water.

The eluate from strip number 1, identified chromatographically as histamine, when tested on the guinea-pig ileum, contained a large amount of histamine so that 0.5 ml. of eluate diluted 1 in 1,000 was equivalent to 0.05 μg. histamine. The eluate activity was completely abolished by mepyramine. Since 5 mg. venom was used for the chromatogram this recovery gives a calculated value of approximately 20 mg. histamine per gram

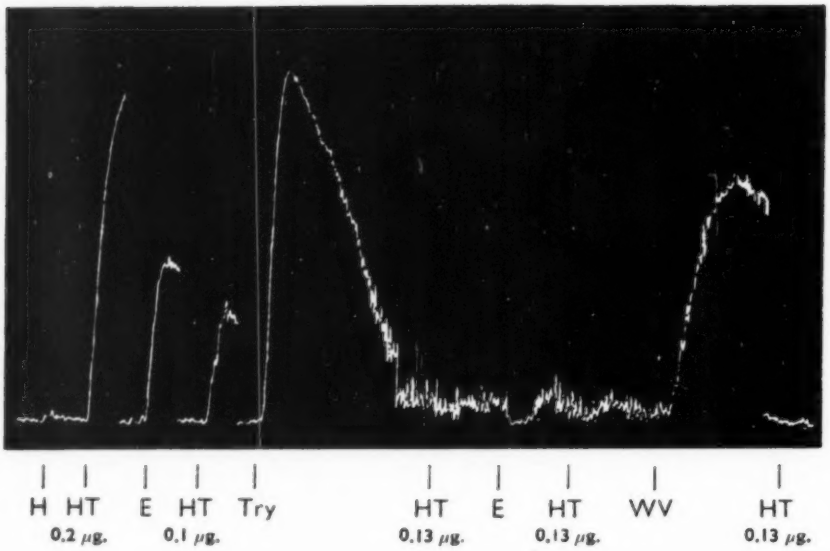

FIG. 3.—Effect on guinea-pig ileum of wasp venom paper chromatogram eluate with R_f value and colour reaction of 5-hydroxytryptamine. Atropine (0·2 μg.) and mepyramine (0·5 μg.) in bath throughout. The eluate is inactive after tryptamine desensitization, whereas untreated wasp venom is still effective. Continuous tracing indicates that solutions were not washed out between tests. H, 0·2 μg. histamine; HT, 5-hydroxytryptamine; E, 0·08 ml. eluate; Try, 200·0 μg. tryptamine; WV, 5·0 μg. wasp venom.

venom. This is in good agreement with the value of 16 mg./g. obtained by biological assay of the venom following Code's method of histamine extraction. It is greater than the histamine concentration (6 mg./g.) found in bee venom by Marcou and Derevici (1937).

The eluate of strip number 3, chromatographically identified as 5-hydroxytryptamine, possessed the pharmacological properties of this substance when tested on the guinea-pig ileum. Its activity was unaffected by mepyramine, but was abolished by tryptamine desensitization of the ileum (Fig. 3). The equivalent of 0.08 ml. of the eluate corresponded in activity to 0.13 μg. of 5-hydroxytryptamine, which yields a calculated value of approximately 0.32 mg. 5-hydroxytryptamine per gram venom. This value, like that of the histamine content of venom obtained from the chromatogram eluate, is a minimal one, since it is possible that some destruction occurs during the formation of the chromatogram and that elution is incomplete.

Histamine Liberating Activity of Wasp Venom

Four experiments were carried out on isolated perfused cat skin preparations to test the ability of wasp venom to release histamine. In each instance, several (2 to 8) venom sacs and their glands were weighed, ground in saline in a glass tissue grinder, centrifuged and the soluble material made up in saline so that 0.5 ml. contained the soluble material of the venom of one wasp. This volume of venom solution was injected intra-arterially into isolated skin in four experiments. Since the venom itself contained histamine, the histamine content of the perfusates was always increased; this was accompanied by a decreased rate of flow—probably largely due to the vascular action of the histamine in the venom. The injected venom was first assayed for its histamine content, and the amount injected subsequently subtracted from the total histamine in the perfusate. In two such experiments the total histamine recovered in the perfusate did not exceed the amount contained in the injected venom. In two others, however, the histamine in the perfusate exceeded the amount injected by 3.2 and 15.4 μg. respectively, indicating that histamine was released from the skin in these instances.

DISCUSSION

Our results demonstrate that wasp venom contains three highly active smooth muscle stimulants —histamine, 5-hydroxytryptamine, and a highly potent unidentified material producing a delayed, slow contraction of the guinea-pig ileum. The average concentration of histamine in the dried venom of a large number of wasps was approximately 16 and 20 mg./g. respectively, as determined by different methods. Since histamine concentrations of 1 in 1,000 cause pain when applied to a blister base in human skin (Armstrong, Dry, Keele, and Markham, 1953), it can be inferred that the injection of histamine by the wasp contributes to the pain and oedema following a wasp sting. Similarly, the minimal possible concentration of 5-hydroxytryptamine in venom was of the order of 0.32 mg./g., as determined by the assay of the eluate from a paper chromatogram. This concen-

tration would also be greater than the pain producing threshold concentration of this substance for human skin (Armstrong *et al.*, 1953). Although the high concentrations of histamine and 5-hydroxytryptamine can account for some features of the human skin reaction following a wasp sting, other factors are undoubtedly involved. For example, our experiments demonstrate the presence in venom of a highly potent substance which produces a delayed, slow contraction of the guinea-pig ileum ; wasp venom is also able, occasionally, to release histamine. Furthermore, the venom contains large amounts of hyaluronidase (Jaques, R., unpublished) which would enhance the diffusion of toxic substances in the skin.

The widespread occurrence of histamine in animal tissues is well established. There is more recent evidence that 5-hydroxytryptamine may also be widely distributed in nature. It has been detected in the mucosa of mammalian gastro-intestinal tract and in spleen (Erspamer, 1940 ; Erspamer and Asero, 1952) ; in blood serum (Rapport, Green, and Page, 1948 ; Rapport, 1949), where its source is the platelets (Rand and Reid, 1951) ; in the salivary glands of octopoda, and in the skin of amphibia (Erspamer and Boretti, 1951). Its presence in wasp venom further emphasizes its ubiquity.

In the preceding paper we have referred to descriptions, by previous workers and by ourselves, of the appearance in plasma of a smooth muscle stimulating substance which gives a characteristically slow contraction of the guinea-pig ileum ; we suggested that different substances might produce this type of contraction. It is of interest, however, that the slow contractor in wasp venom has a similar action on the guinea-pig ileum to the substance which appears in cat plasma after injection of thalassine. Analysis of this constituent in wasp venom revealed several points of similarity to bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949), the slow contracting substance obtained by incubating globulin with snake venom or trypsin, and which may be a polypeptide produced by proteolytic breakdown of globulin. The substance in wasp venom withstands boiling for long periods at neutral—though not at high—*p*H ; contracts the rat colon—which is, however, less sensitive to it than the guinea-pig ileum—and is inactivated when the venom is treated by Code's method for histamine extraction. In this last, it is probably the boiling with concentrated hydrochloric acid that destroys the activity. All these properties have also been described by Rocha e Silva and his co-workers for bradykinin. The most effective purified bradykinin preparation they obtained produced strong contractions of the guinea-pig ileum in concentrations of 1 in 1–3,000,000. But crude, untreated wasp venom often produces a marked contraction in concentrations of 1 in 3,000,000 on a preparation rendered insensitive to acetylcholine, histamine and 5-hydroxytryptamine. Thus, crude wasp venom has the same order of potency as has a highly purified bradykinin preparation. The substance in crude venom producing this effect must be highly active. If, for example, it comprises 1% of the total wasp gland its potency must be as great as that of histamine.

It is well established that histamine is released in anaphylaxis. Recently, the release of bradykinin (Beraldo, 1950) and 5-hydroxytryptamine (Humphrey and Jaques, 1953) have also been demonstrated during this reaction. It is a curious fact that wasp venom contains large amounts of histamine, 5-hydroxytryptamine and a substance that resembles bradykinin.

Summary

1. Histamine, 5-hydroxytryptamine, and a potent unidentified smooth muscle stimulant were found in high concentrations in the venom of the common wasp.

2. Histamine was identified in untreated venom by tests on the isolated guinea-pig ileum ; the histamine content of pooled wasp venom was approximately 16 mg./g. venom following Code's method of extraction. Histamine was also identified in venom by paper chromatography ; bio-assay of the specific eluate gave a histamine content of approximately 20 mg./g.

3. 5-Hydroxytryptamine was identified in wasp venom by paper chromatography and the specific eluate found to be pharmacologically identical with this substance. Pooled wasp venom had a 5-hydroxytryptamine content of approximately 0.32 mg./g.

4. A potent, unidentified substance producing a delayed slow contraction of the guinea-pig ileum was also present in venom. The properties of this substance were similar to those described for bradykinin.

5. Wasp venom occasionally released histamine from perfused cat skin.

We wish to thank Dr. R. K. Richards of Abbott Laboratories, North Chicago, for the supply of serotonin creatinine sulphate.

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Brit. J. Pharmacol. (1954), **9**, 59.

STOOL CONCENTRATIONS AND ABSORPTION OF CHLORAMPHENICOL AND ITS PALMITATE IN BABIES

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For a period after its introduction chloramphenicol was widely used in the treatment of enteral infections because of its bacteriostatic action against a wide range of Gram-negative organisms. But reports of its efficacy in the treatment of some of these infections, especially infantile gastro-enteritis, have been conflicting (Rogers, Koegler, and Gerrard, 1949; Smellie, 1950; Cerruti and Scarzella, 1950; Shanks and Studzinski, 1952). Stool concentrations have, therefore, been assayed in a series of infants under treatment in the hope that the results might lead to a better understanding of the role of chloramphenicol in treatment. Welch (1950) has made a few studies on normal adults, but these are not necessarily applicable to infants.

Early in this study it became apparent that chloramphenicol palmitate differed from the crystalline form in its pharmacological behaviour. The palmitate, which was introduced primarily as a palatable form for children, is an ester which contains 57.6% by weight of chloramphenicol and possesses practically no antibiotic activity. Its action depends on the release of the free alcoholic form by the intestinal lipases (Ross, Burke, and Rice, 1952). In view of this difference in behaviour two series of infants were treated, one with the crystalline form and one with the palmitate, and the stool and serum levels of each group were compared. Because of its potential toxicity the popularity of chloramphenicol has fallen, but an annotation in the *Lancet* (1953) gives a guide to its use by stating that "in moderate dosage and for short administration it is safe enough."

MATERIAL AND METHODS

Clinical Material.—25 infants, of ages ranging up to 15 months, were studied. They suffered from a variety of conditions, and some had loose stools.

Dosage.—Infants on crystalline chloramphenicol were given 160 mg./kg./day divided into four doses. Those on palmitate received a dose which could release 160 mg./kg./day of active chloramphenicol from the ester (4 ml. of suspension is equivalent to 125 mg. of crystalline chloramphenicol).

Sampling Methods.—Venous blood and stools were obtained at approximately the same time each day, after the infants had been on treatment for at least 24 hr. Stools were obtained by introducing a short sterilized rubber tube into the rectum. If the bowel contents were fluid they flowed out easily. If formed, the rectal reflex, excited by the passage of the tube, caused the early passage of a specimen. Stools were thus obtained fresh and uncontaminated.

24-hr. urine specimens were collected from six male babies, through a sterilized system, for assay of urinary excretion. Collection was not begun until at least 24 hr. after the start of treatment. Fluid intake was standardized according to body weight, and no case showed evidence of fluid imbalance.

Assay Methods.—The following preliminary treatment of the specimens was always carried out within half an hour of completing the collection.

1. *Blood Serum.* Venous blood, which had been withdrawn under sterile conditions, was allowed to clot in a plain sterile tube. The specimen was then centrifuged, and the supernatant serum removed by pipette and transferred to another plain sterile tube. This tube was then incubated at 56° C. for 30 min. to inactivate enzymes which might destroy the chloramphenicol.

2. *Stool.* 2 g. of moist stool (in some instances this was fluid) was emulsified with 10 ml. of peptone water and boiled in a water-bath for exactly 20 min. This process inactivated the enzymes present and sterilized the specimen. The emulsion was then centrifuged to yield a supernatant fluid suitable for Seitz filtering. This fluid was transferred to the chamber of a centrifuge Seitz filter, and a clear sterile filtrate suitable for assay was obtained.

3. *Urine.* These specimens were inactivated by incubating at 56° C. for 30 min. and Seitz filtered before assay.

On a few occasions it was inconvenient to start immediate assay. The fluid was therefore stored in a refrigerator until the actual assay was carried out.

Serial double dilutions in peptone water of the fluid for assay were set up in 3-in. × ⅛-in. test-tubes with a final volume of 1 ml. in each tube. Two tubes, one for growth control and one for sterility control, were added. The tubes were inoculated with a 2.5-mm. loopful of an overnight broth culture of El Tor vibrio and incubated overnight. The highest dilution to show no growth was read as containing 0.5 μg./ml. of chloramphenicol and the original concentration calculated from this. For example, in the following experiment there was no growth in tube 3 and growth in tube 4:

Tube ...	1	2	3	4	5	6
Dilution ...	1/2	1/4	1/8	1/16	1/32	1/64
Growth ...	−	−	−	+	+	+

The concentration in tube 3 is taken as 0.5 μg./ml., and as the contents of this tube have been diluted eight times the original concentration will be (8 × 0.5) or 4 μg./ml. Although the accepted method is to record the concentration as 4 μg./ml., it is apparent that the actual concentration lies between 4 and 8 μg./ml.

By using a different method of diluting the fluid for assay, in which the differences in dilutions were much less, it was possible to reach a more accurate end-point. This method was used to assay the urine concentrations and to test the sensitivity of the organism.

The vibrio was tested at intervals and found to remain constantly sensitive to 0.5 μg./ml. and insensitive to 0.4 μg./ml. of chloramphenicol. The accuracy of the method was tested by assaying normal stools with a known amount of chloramphenicol added. Six normal stools were also assayed and found to have no recordable antibiotic activity.

It was not possible to record stool levels below 6 μg./g. because of the necessary dilution of the stools with peptone water.

Results

Serum and Stool Levels.—The serum levels are compared in Table I and the stool levels in Table II. These indicate that chloramphenicol palmitate yields lower serum levels and higher, though scattered, stool levels than does the crystalline form.

The mean value of each group of serum and stool levels has been calculated to compare these differences more easily. As the recorded levels in each group rise in geometric progression (as a result of the double dilutions) the geometric mean of each group was calculated according to the formula:

$$\text{Log } G = \frac{(\log x_1 + \log x_2 + \ldots\ldots + \log x_n)}{N}$$

where G is the geometric mean.

N is the number of values in the series and $x_1, x_2 \ldots\ldots x_n$ are the individual values. It was pointed out previously how, although a value is recorded as 4 μg./ml., it lies in reality between 4 and 8 μg./ml. For the purposes of mathematical accuracy this value is taken as the geometric mean between 4 and 8, i.e., 5.7 μg./ml. Thus each individual value in the above series $x_1, x_2 \ldots\ldots x_n$ is taken as the geometric mean between it and the next highest value. Values of less than 6 μg./g. presented a problem, as it is not possible to calculate a geometric mean between 0 and 6. The arithmetic mean of 3 was assumed as a reasonable figure. Thus on a comparable dosage the mean levels are:

(*a*) Serum. On chloramphenicol palmitate, 7.1 μg./ml. On crystalline chloramphenicol, 18.6 μg./ml.

A t-test shows that the difference between the means of the two samples is highly significant ($P<0.01$).

(*b*) Stools. On chloramphenicol palmitate, 33.1 μg./g. On crystalline chloramphenicol, <6 μg./g.

There is again a highly significant difference between the means of the two samples.

As a test of the consistency and reliability of the serum and stool findings, it was found possible, by assaying these levels in individual babies, to decide which form of chloramphenicol was being given. In addition, in four babies a change from one form of drug to the other was always reflected in an appropriate change in the serum and stool levels.

Urine Levels.—The percentage of the 24-hr. oral dose excreted in the urine in the same period was 10, 7 and 6% respectively in three babies on

TABLE I

SERUM LEVELS OF CHLORAMPHENICOL IN TWO GROUPS OF BABIES TREATED WITH COMPARABLE DOSES OF THE CRYSTALLINE AND PALMITATE FORMS

Form of Chloramphenicol	No. of Cases with Serum Levels per ml. of				
	2 μg.	4 μg.	8 μg.	16 μg.	32 μg.
Crystalline ..	0	1	3	9	1
Palmitate ..	2	9	6	1	0

TABLE II

STOOL LEVELS OF CHLORAMPHENICOL IN TWO GROUPS OF BABIES TREATED WITH COMPARABLE DOSES OF THE CRYSTALLINE AND PALMITATE FORMS

Form of Chloramphenicol	No. of Cases with Moist Stool Levels per g. of							
	< 6 μg.	6 μg.	12 μg.	24 μg.	48 μg.	96 μg.	192 μg.	384 μg.
Crystalline ..	14	1	1	0	0	0	0	0
Palmitate	2	5	5	5	4	3	1	2

crystalline chloramphenicol, and 4.1, 2.3 and 1.2% in three babies on chloramphenicol palmitate.

Urine levels closely reflect serum levels. This small experiment served to check whether, in fact, higher urine levels did occur in the group on crystalline chloramphenicol as compared with that on the palmitate. Though the results are too few for statistical analysis they do suggest a higher urinary excretion of the crystalline form. They therefore add weight to the serum and stool findings.

Discussion

These results, taken in conjunction with published serum absorption curves (Ross, Burke, and Rice, 1952; O'Brien, 1953), indicate that crystalline chloramphenicol is effectively absorbed from the bowel giving high serum and urine levels. In only two out of sixteen cases was it possible to record stool concentrations above 6 μg./g. It may be, however, that a proportion of the drug is excreted in an inactive form, since it is known that intestinal and bacterial enzymes may inactivate it.

The palmitate, in contrast, yields low serum and urine levels and high though widely scattered stool levels.

These findings have practical applications which may be summarized as follows:

(1) Where a pathogen lies in the body tissues or urinary tract, then, if chloramphenicol is the drug of choice, the crystalline form should be used.

(2) Where a pathogen lies in the lumen of the bowel, the palmitate is preferable. In this situation, however, the antibiotic of choice should be bactericidal in action.

Summary

In comparable doses, in babies, chloramphenicol palmitate yields lower serum and urine levels and higher stool levels than does the crystalline form.

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THE EFFECT OF INTRAVENOUS INFUSIONS OF HISTAMINE ON THE URINARY HISTAMINE AND ON GASTRIC SECRETION IN MAN

BY

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When histamine is infused intravenously in man at a rate that produces distinct pharmacological effects, it can be easily detected in the urine but not in the venous plasma (Adam, 1950). This author estimated the urinary histamine by a simplified method based on that described by Anrep, Ayadi, Barsoum, Smith, and Talaat (1944). It was possible by this method to follow the excretion when 3.3 mg. of histamine base was infused intravenously, but not the normal or basal excretion of free histamine in the urine. Roberts and Adam (1950) have since shown by a new method that free histamine appears continuously and in measurable amounts in the urine of healthy men, and suggested that the immediate source of this histamine was the blood plasma. The aim of the present work was to obtain further evidence in support of this assumption by following the effect of intravenous infusions of histamine on the urinary excretion of free histamine and on the acid gastric secretion. It was possible in this way to measure the proportion of the drug excreted during the infusion of small doses and to relate the amount excreted to the effect of the infusion on the acid gastric secretion. Since it was not possible to detect free histamine in the plasma by direct methods, the acid gastric secretion was used as a measure of the concentration of free histamine in the circulating blood (Ivy and Javois, 1924 ; Kalk, 1929 ; Ungar, 1935 ; Feldberg and Holmes, 1941 ; Emmelin, Kahlson, and Wicksell, 1941 ; Grob, Lilienthal, and Harvey, 1947).

Incidental observations were made on the histamine content of the gastric juice. The dose-response relationship of histamine to the acid secretion of the stomach was also investigated, but the results will be reported elsewhere.

Three healthy men, designated as W.I.C., J.A.S., and A.A.G., each received a series of histamine infusions, and the free histamine was estimated in samples of the urine, venous blood plasma and gastric juice. The doses infused were in the range that produced graded effects on the acid gastric secretion. Infusions with the lower doses were symptomless, yet they produced a distinct stimulation of the acid secretion and a measurable increase in the excretion of histamine in the urine.

METHODS

Pharmacological Methods

Extraction of Free Histamine.—The method of Roberts and Adam (1950) was used, and their paper should be consulted for the precise details. This method consists in the separation of free histamine from conjugated histamine and other substances in 50 to 100 ml. of urine by absorption on a column of the cationic exchanger Decalso F (The Permutit Co., London). The histamine is then eluted by treating the column with concentrated ammonium hydroxide (A.R.) followed by chloroform saturated with ammonia gas. After evaporation of the eluent, the extract is taken up in 5 to 10 ml. 0.9% NaCl and neutralized. In this way it is possible to concentrate the free histamine to the extent required for the biological assay. The mean recovery of 116 experiments in which histamine was added to various body fluids in the range of 0.5 to 5 μg. was 67% $\pm$1.1 (S.E. of mean). This fraction is a characteristic of the Decalso column, and a correction can be applied to the results ; the results presented in this paper are uncorrected. The same method, with slight modifications, was used for the extraction of histamine from venous blood plasma and gastric juice. Some of the 25-ml. plasma samples were divided and 10 ml. extracted in parallel by Code's modification (1937) of the method of Barsoum and Gaddum (1935). The Decalso method does not require precipitation of the plasma proteins, but, unlike the urine, the plasma extract must be boiled in strong acid to destroy other gut-contracting substances. The plasma samples were concentrated three- to five-fold by taking up the extracts in 5 ml. 0.9% NaCl. An aliquot of 25 ml. from a pooled sample of gastric juice was filtered through glass wool, and mucus in the filtrate precipitated at *p*H 4 by the addition of an equal volume

of acetone. After removal of the precipitate by centrifuging, the supernatant fluid was brought to *p*H 8, recentrifuged and applied to the column. Like the plasma, the gastric juice extract was boiled in strong HCl to destroy other substances which interfere with the assay. The final extract was taken up in 5 ml. 0.9% NaCl.

Estimation and Identification of Histamine.—The histamine values are all calculated in terms of the base, on the assumption that this represents 36.16% of the weight of the phosphate. The extracts were tested on a strip of guinea-pig ileum suspended in 2 ml. Tyrode's solution containing atropine (0.1 μg./ml.) and were compared with a standard solution of histamine acid phosphate (British Drug Houses Ltd.). Mepyramine maleate (May and Baker Ltd.) was used as a test for histamine in some of the extracts (Reuse, 1948).

Clinical Methods

Most of the infusions were given at weekly intervals. A preliminary infusion of 0.9% NaCl preceded each infusion of histamine. The histamine infusion itself lasted about three hours, and during this time the drug entered the circulation at a constant rate. Samples of urine, venous blood, and gastric juice were collected before and during the infusion; urine was also collected after the infusion. In a separate experiment each subject received a control infusion of physiological saline only; this was also maintained for three hours.

Collection of Samples. Urine.—This was collected in chemically clean stoppered flasks of 750 ml. capacity which contained 2 to 5 ml. 2N HCl to reduce the *p*H of the urine to less than 4. The samples were stored at 0° C. pending extraction of the histamine two to three hours later.

At a definite time before the preliminary infusion of saline was due to start the subject emptied his bladder and discarded the urine. Immediately before the infusion of histamine was begun he emptied it again to provide the first control sample. At the end of the infusion he again passed urine: this middle sample contained all urine formed during the infusion plus a small amount that was formed after the infusion was stopped. There was always some delay in collecting this sample because it was essential to free the subject from the apparatus. The average delay was 14 min. (range (18) 8–35 min.). A second control sample was collected in the course of the next three hours.

A correction for the basal excretion of free histamine was applied to the values obtained from urine samples formed during the infusion. It was assumed, on reasonable evidence (see Table II), that excretion of the dose in the urine ceased soon after the infusion was ended. This simplified the calculation, which was done in the following way. The sum of the histamine values for the control samples was divided by the total time in minutes required for the collection of these samples. The result gave the mean rate of excretion in μg./min. during the control periods. The product of this rate, and the time in minutes over which the middle sample was collected, provided an estimate of the basal excretion during the infusion. The difference between the amount of free histamine in the sample formed during the infusion and the calculated basal excretion was taken to represent the amount of administered histamine that appeared in the urine; this amount was expressed as a percentage of the dose infused.

Venous Blood.—Two samples were withdrawn from each subject at the time he received the largest dose of histamine: the first sample was obtained during the preliminary saline infusion, and the second towards the end of the histamine infusion. A needle (15 S.W.G.) with rubber tubing attached was inserted into the antecubital vein and 50 ml. of blood allowed to run into a glass tube containing 500 units of heparin (Liquemin, Roche). The sample was kept at 2–4° C. for about an hour and then centrifuged at 3,000 r.p.m. for 15 min. The plasma was separated and applied to the column without delay.

Gastric Juice.—The subject omitted breakfast, and, about two hours before the infusion, a Ryle's tube was passed into the stomach and its position verified radiologically. He then lay in a semi-recumbent position and the tube was connected to a source of continuous suction. The collection of 10-min. samples was begun 40 to 120 min. before, and continued throughout, the infusion. The free and total acid were determined in each sample by titration with N/10 sodium hydroxide, first to Töpfer's reagent and then to phenolphthalein as indicator. The summed values for the total acid output* only are given in this paper, and are expressed in milli-equivalents of HCl. The samples collected during the control and infusion periods were pooled independently. An aliquot of 25 ml. was taken from each pooled sample for estimation of the free histamine. Estimates were calculated for the basal amounts of histamine and total acid in the gastric juice, and a correction was applied to the results in the manner already described for the urine.

Intravenous Infusion of Histamine.—A sterile stock solution was prepared on the day before infusion, from which a dilution with 0.9% NaCl was made for infusion. A 50-ml. glass syringe was filled with this solution and fixed horizontally in an apparatus which drove the piston forward at a constant rate. The mean volume flow from the needle connected to the syringe as for infusion was 32.2 ± 0.3 ml. per hour. The syringe did not usually require to be refilled more than once during the infusion, and the delay involved was less than two minutes.

RESULTS

These have been summarized for each subject and are set out in Table I. The data for the urine are given in Table II. The experiments are numbered in order of magnitude of the dose.

* Total acid output = concn. in m.equiv./l. × volume in litres.

TABLE I

HISTAMINE VALUES IN URINE, GASTRIC JUICE AND BLOOD PLASMA DURING INTRAVENOUS INFUSION OF HISTAMINE ACID PHOSPHATE, IN THREE SUBJECTS

(All the histamine values are expressed as the base.)

Exp.	Infusion			Urine				Gastric Juice						Blood Plasma	
	Duration min.	Dose (μg.)	Rate ng./kg./min.	Basal (calc.) μg.	Total (μg.)	Difference (μg.)	Difference Expressed as % of Dose	Total Acid Output (m.equiv. HCl)	Basal (calc.) μg.	Basal (calc.) μg./l.	Total μg.	Total μg./l.	Difference (Total/Basal) μg.	Control μg./l.	Infusion μg./l.
							Subject W.I.C. ♂ 82 kg.								
1	181	0	0	0·9	1·1	0·2		−1·2							
2	191	206	13·1	1·5	3·6	2·1	1·0	6·0	3·8	14	4·6	20	0·8		
2a	165	176	13·0	0·6	2·1	1·5	0·8								
3	217	521	29·1	1·9	7·2	5·3	1·0	18·1	12·6	20	9·3	22	−3·3		
4	180	775	52·2	1·7	11·1	9·4	1·2	33·9	2·0	12	10·6	26	8·6		
5	172	1,650	116·2	2·4	22·3	19·9	1·2	49·5	2·7	21	16·3	31	13·6	<3	<3
							Subject J.A.S. ♂ 75 kg.								
1	170	0	0	1·8	1·5	−0·3		2·3	0·04	0·8	1·4	20	1·4		
2	162	44	3·6	1·6	2·1	0·5	1·1	4·8	0·8	14·0	0·5	6	−0·3		
3	175	105	8·1	1·5	2·9	1·4	1·3	6·6	0·2	3·5	0·8	8	0·6		
4	180	216	16·0	1·9	3·7	1·8	0·8	20·7	1·6	15·5	5·0	20	3·4		
5	181	390	29·0	2·3	6·0	3·7	0·9	55·5	—	—	—	—	—		
6	175	630	48·0	1·7	6·3	4·6	0·7	72·5	2·8	34·5	5·2	9	2·4		
7	182	980	72·0	2·5	12·1	9·6	1·0	87·2	0·7	12·5	1·6	3	0·9	<3	<3
							Subject A.A.G. ♂ 73 kg.								
1	177	0	0	1·1	1·2	0·1		0·7							
2	183	98	7·4	1·3	3·2	1·9	1·9	5·9							
3	185	197	14·8	1·1	3·9	2·8	1·4	22·4							
4	185	395	29·6	1·7	6·4	4·7	1·2	29·1							
5	182	777	59·2	0·8	15·1	14·3	1·8	38·9						<3	<3

In none of the three subjects did the control infusion of physiological saline produce an important rise in the urinary histamine, and the effect on the acid secretion was negligible. The values for the excretion before the histamine infusion show only small differences when compared with those after the infusion (Table II). In five out of seven infusions (Nos. 1, 2, 4, 5 and 7) J.A.S. excreted histamine at a slightly faster rate after the infusion than just before it: but it is improbable that he was still excreting infused histamine, since the effect did not increase with the dose, and also occurred after the infusion of saline alone. In two experiments on W.I.C. (Nos. 2 and 2a) exceptionally high values were obtained in samples collected after the infusion: we are unable to explain these particular results—which may have been due to some fault in technique—and have therefore disregarded them in our calculations. With these exceptions, it seems probable that the excretion of histamine given by infusion did not continue appreciably beyond the period of the infusion.

In many of the experiments the urine sample collected after the infusion was very concentrated. This was also seen in the control infusions with saline and was probably an effect of the prolonged fast and the continuous removal of gastric juice.

The calculated values for the basal excretion during the infusion were remarkably close in the different experiments on the same subject and even between subjects. It was thus possible to detect small increases when histamine was infused at low rates. The result obtained by infusing histamine at a rate of 3.6 ng./kg./min. (Table I, J.A.S., Expt. 2) is doubtful, but infusions at rates of 7.4 and 8.1 ng./kg./min. (Table I, A.A.G., Expt. 2; J.A.S., Expt. 3) about doubled the excretion of free histamine in the urine and also stimulated gastric secretion. In each of the three series of infusions, the excretion of histamine in the urine and the effect on the acid gastric secretion increased with the dose. The relation between the dose of histamine and these effects is also shown graphically (Fig. 1) for one series of infusions. In all these experiments the proportion of histamine excreted was approximately 1% of the dose (range (15) 0.7–1.9%) and this proportion was independent of the infusion rate.

Infusions up to a rate of 30 ng./kg./min. were indistinguishable in their subjective effects from the control saline infusion. Headache occurred neither during nor after these infusions and there was no flush or tachycardia; the only effect, apparently, was the stimulation of gastric secretion.

Even when the infusion rate was high (Table I, W.I.C., Expt. 5; J.A.S., Expt. 7; A.A.G., Expt. 5) histamine was not detected in samples of the venous blood plasma either by the Decalso or by Code's method. Since the Decalso method allowed of a three-fold concentration of any free histamine that might have been present in the plasma, it was possible to conclude from the assay that the hist-

TABLE II

DATA ON URINARY EXCRETION OF HISTAMINE BEFORE, DURING, AND AFTER INTRAVENOUS INFUSION, IN THREE SUBJECTS

Histamine is expressed as base

Expt.	Sample	Collection Time (min.)	Vol. of Urine (ml.)	Histamine (μg.)
	Subject W.I.C.			
1	Before infusion	153	104	0·83
	During ,,	192	184	1·10
	After ,,	170	54	0·72
2	Before ,,	140	207	1·0
	During ,,	207	200	3·6
	After ,,	148	36	(8·3)
2a	Before ,,	156	102	0·51
	During ,,	190	303	2·14
	After ,,	204	101	(7·7)
3	Before ,,	227	554	2·2
	During ,,	230	180	7·2
	After ,,	215	740	1·5
4	Before ,,	135	86	1·2
	During ,,	200	238	11·1
	After ,,	185	73	1·5
5	Before ,,	150	150	1·4
	During ,,	210	290	22·3
	After ,,	160	98	2·2
6	Before ,,			
	During ,,	—	—	—
	After ,,			
	Subject J.A.S.			
1	Before ,,	190	245	1·1
	During ,,	192	185	1·5
	After ,,	170	78	2·5
2	Before ,,	204	216	1·7
	During ,,	171	148	2·1
	After ,,	165	56	1·8
3	Before ,,	150	293	1·3
	During ,,	185	184	2·9
	After ,,	150	61	1·1
4	Before ,,	137	127	1·0
	During ,,	190	208	3·7
	After ,,	148	53	1·9
5	Before ,,	160	250	1·4
	During ,,	200	250	6·0
	After ,,	195	134	2·7
6	Before ,,	165	175	1·4
	During ,,	195	97	6·3
	After ,,	180	48	1·5
7	Before ,,	180	173	1·7
	During ,,	200	151	12·1
	After ,,	210	120(?)	3·2
	Subject A.A.G.			
1	Before ,,	135	155	0·78
	During ,,	188	310	1·24
	After ,,	187	30	1·12
2	Before ,,	167	155	1·24
	During ,,	190	308	3·23
	After ,,	245	82	1·64
3	Before ,,	160	465	0·93
	During ,,	193	430	3·90
	After ,,	172	79	0·92
4	Before ,,	140	74	1·04
	During ,,	192	138	6·4
	After ,,	180	80	1·87
5	Before ,,	152	238	0·95
	During ,,	191	360	15·10
	After ,,	192	54	0·40

amine in the plasma was less than 3 μg./l. Nevertheless, the effect of the infusion on the acid secretion was always pronounced and the corresponding increase in the urinary free histamine (total/basal) was 9, 5, and 19-fold respectively in the three experiments.

The histamine content of the gastric juice was estimated in only two of the subjects (Table I,

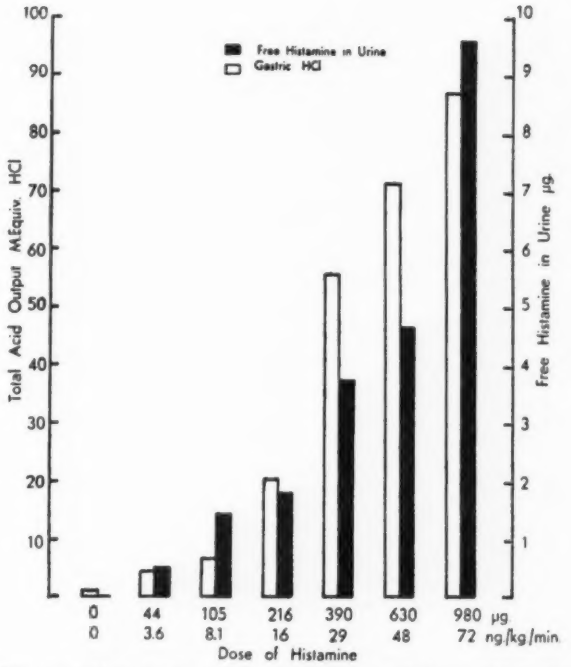

FIG. 1.—Subject J.A.S. Effect of intravenous infusions of histamine on acid gastric secretion and on urinary excretion of free histamine. The duration of each infusion was about 180 min. The dose is shown as the total amount infused (μg.) and as the rate of infusion (ng./kg./min.).

W.I.C., J.A.S.). Free histamine was present in the normal gastric juice under resting conditions, but the quantities varied widely. When the flow was stimulated by the infusion of histamine, the total amount of histamine in the juice increased in 7 out of 9 experiments, but the increase bore no close relationship to the dose infused. The concentration of histamine rose slightly in all but three experiments (Table I, J.A.S., Expts. 2, 6, and 7).

DISCUSSION

Little is known about the fate of histamine injected into the blood stream. The results obtained in the present experiments, and those previously reported (Adam, 1950) indicate that about 1% of the dose is excreted in the urine in the free form and that none is conjugated. The remaining 99% disappears in other ways, possibly by combination with an enzyme system similar to histaminase (Best and McHenry, 1930) or with tissues upon which it has no action (Gaddum, 1948).

Within the limits of the doses used in these experiments, the proportion that appeared in the urine was independent of the rate of infusion; it may also be independent of the concentration of histamine in the plasma (Teorell, 1933; Öbrink, 1948). Roberts and Adam (1950) have shown that free histamine appears continuously in the urine

and suggested that the plasma was the immediate source of this histamine. The results support this hypothesis, but, owing to the failure to detect histamine in the plasma, the evidence is indirect. The recovery of 1% of the dose in the urine suggests that the normal excretion (approximately 20 μg./24 hr.) represents a similar proportion of histamine that may be continuously entering the blood stream —either as a result of metabolic processes in the tissues, or of absorption from the alimentary tract, or of both (Gaddum, 1951). Calculation shows that this quantity would be of the order of 2,000 μg./24 hr., or roughly 20 ng./kg./min. for a man of 70 kg., which is within the range of infusion rates used.

When histamine is infused intravenously at a constant rate of about 10 ng./kg./min., the acid secretion is slightly stimulated and the excretion of histamine is measurably increased. On infusing still larger doses, the effect on the acid output and on the urinary excretion increases correspondingly. Headache and other effects on the circulation occurred only when the rate of infusion exceeded 30 ng./kg./min. It therefore seems possible that even slight changes in the plasma histamine concentration can be detected indirectly by determining the urinary free histamine. This suggests that the liberation of histamine in the tissues or its absorption from the gut may be studied by following its excretion in the urine. Such a method, however, would be more likely to detect the slow continuous passage of histamine into the blood stream than it would the sudden release of a large quantity which might disappear in other ways before an appreciable volume of urine could be formed.

When histamine is given by slow intravenous infusion it disappears rapidly from the blood (Emmelin, 1951) and this contributes to the difficulty of estimating it in the plasma, particularly when the dose is small. Our failure to detect histamine activity in the plasma may be explained in several ways. Histamine may have disappeared from the plasma before this was separated from the cells. However, it is also possible that some of the plasma histamine is loosely bound to proteins (Emmelin, 1945 ; Öbrink, 1948) or conjugated (Anrep *et al.*, 1944). By the Decalso method, it may be expected that these pharmacologically inactive forms of histamine would be lost in the percolate. If the plasma histamine consisted mainly of these forms, the quantity of histamine retained on the column as the cation and subsequently eluted as the free base would probably be less than could be detected by the method of assay. But the evidence for this view is inconclusive, since the results obtained by Code's method, which would be expected to measure the total plasma histamine, were also negative. Again, blood sampled at the antecubital vein may not have contained free histamine. This would be so if the dose infused per minute disappeared during a single passage of the blood through the capillaries. Some of these problems are at present being investigated, and until more data are obtained further discussion is unprofitable.

Free histamine is present in measurable amounts in the resting gastric juice, where its concentration is similar to that in the urine but more variable. The slight increase in concentration observed in six out of nine histamine infusions agrees with findings in the cat (Emmelin and Kahlson, 1944) and in the dog (Code, Hallenbeck, and Gregory, 1947). Emmelin and Kahlson concluded that the parietal cells are permeable to histamine given in this way. It is, however, difficult to explain this effect in man by simple diffusion, since the concentration of free histamine in the plasma was lower than in the gastric juice. Whether this histamine derives from the plasma or from the gastric mucosa (Trach, Code, and Wangensteen, 1944) cannot be decided on the present evidence.

Summary

1. The effect on the urinary free histamine, and on the acid gastric secretion, of histamine given by slow intravenous infusion has been studied in three healthy men.

2. When histamine was infused at a rate that stimulated acid secretion there was a corresponding rise in the excretion of free histamine in the urine. The threshold rate for both of these effects varied from 7.4 to 13.0 ng./kg./min.

3. The proportion of free histamine that appeared in the urine during the infusion was about 1% of the dose and was independent of the rate of infusion.

4. There was no detectable increase in the histamine content of plasma obtained from the antecubital vein during the course of infusions which stimulated the acid gastric secretion.

5. Free histamine was present in the resting gastric juice, where its concentration was similar to that in the urine but more variable. The amount appearing in the juice bore no close relation to the rate of infusion.

6. The evidence obtained supports the view that the free histamine normally excreted in the urine derives from the plasma.

We are grateful to Dr. A. A. Guild, of the Department of Medicine, who volunteered to act as a subject in these experiments.

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THE ROLE OF THE CYCLIC ETHYLENEIMINIUM ION IN THE PHARMACOLOGICAL ACTIVITY OF THE 2-HALOETHYLAMINES

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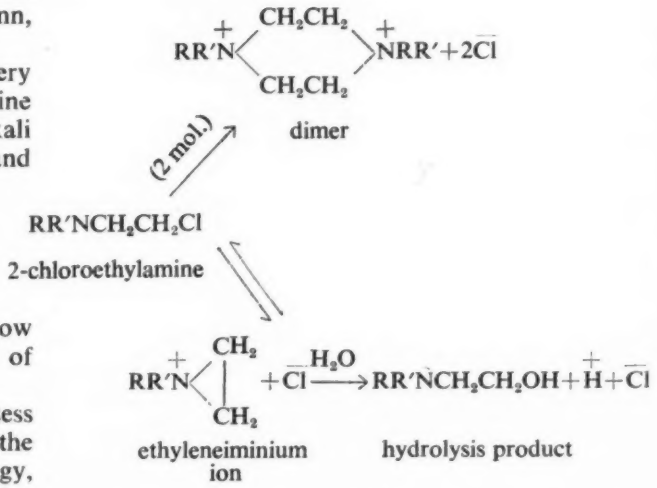
The sulphur and nitrogen "mustard" derivatives, to which the 2-halogenoethylamines are chemically related, form intermediate compounds which may react with constituents of biological systems in aqueous solution at physiological *p*H. The basic chemical reaction to which the nitrogen and sulphur "mustards" owe their specific activity is probably cyclization leading to the formation of N-substituted ethyleneiminium cations and halogen ions (Golumbic, Fruton, and Bergmann, 1946).

The ethyleneiminium ion produced is very reactive, re-forming the original haloethylamine in the presence of halogen hydracid, while alkali may convert it either to the dimer (Hanby and Rydon, 1947) or to the half-hydrolysis product. The latter can again become an ethyleneiminium ion by the cyclization of the remaining chloroethylamine group. Ethyleneiminium ions react rapidly with sodium thiosulphate and the reaction is now in general use for the estimation of this type of compound in a mixture of products.

Unlike the nitrogen "mustards," which possess two 2-chloroethylamine groups, compounds of the dibenamine type possess only one. By analogy, the latter were assumed to undergo similar changes *in vivo* to those known to be undergone by the nitrogen "mustards" *in vitro* (Nickerson, Nomaguchi, and Goodman, 1946; Nickerson and Goodman, 1948). The antiadrenaline activity was attributed to the ethyleneiminium ion, which was presumed to be formed.

Thus compounds of the dibenamine type were assumed to undergo the changes in neutral aqueous media as shown in the next column.

In the light of this hypothesis, Nickerson and Gump (1949) examined a large number of 2-haloethylamines and related compounds in an attempt to find the structural features necessary to produce antiadrenaline action in this series. Intermediate products such as dimers were prepared and found to be inactive, as also were the alcoholic end products. It was therefore assumed that the intermediate cyclic ethyleneiminium ion was the pharmacologically active species.



Chapman, James, Graham, and Lewis (1952) have shown that ethyleneiminium ions are in fact formed from haloethylamines of the dibenamine type, and Chapman *et al.* (1952) have isolated a picrylsulphonate of such an ion. 2-Fluoro-ethylamines of this type are, however, exceptional: they do not give rise to ethyleneiminium ions, whereas the corresponding chloro, bromo and iodo compounds do so readily; nor do they display the antiadrenaline and antihistamine activities so characteristic of the corresponding chloro, bromo and iodo compounds (Chapman and James, 1953; Graham and Lewis, 1953). Consequently, it

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appears that the antiadrenaline and antihistamine activities of this group of compounds are due, not to the haloethylamines themselves, but to the ethyleneiminium ions formed from them.

The purpose of this investigation was to test this assumption further and to discover whether other less specific activities were due to the unchanged 2-haloethylamines themselves.

METHODS

1. *Chemical.*—The chemical investigation was carried out by Chapman and James (Chapman, James, Graham, and Lewis, 1952). This consisted of determination of the concentration of ethyleneiminium ion, by means of sodium thiosulphate titration, in aged solutions of the four 2-haloethylamines selected, viz.: J10, J11, J12, and J13, i.e., N-α-naphthylmethyl-N-ethyl-2-haloethylamine in which the halogen was Cl, Br, I, and F respectively. A neutral aqueous-acetone solution was employed, the composition of which is given below. Curves were obtained relating the concentration of ethyleneiminium ion with age of the neutral solution. The amounts of halide ion formed in cyclization and of the hydrogen ion formed in hydrolysis of the iminium ion were also determined.

2. *Pharmacological.*—The solutions used were prepared as follows:—The compound (0.0002 moles) was dissolved in 2.5 ml. of glass-distilled water, 6 ml. of acetone added, and the solution brought to 30° C. 1 ml. of 0.2 N-sodium hydroxide was added, and the volume adjusted to 10 ml. with distilled water. This solution was maintained at a temperature of 30° C. throughout the experiment.

At various intervals of time samples were taken, diluted with saline and immediately assayed; similar solutions without the haloethylamine were used as controls. The ED99 against the pressor response to adrenaline (4 μg./kg.) in spinal cats and the ED75 against the depressor responses to histamine (2 μg./kg.) in cats anaesthetized with chloralose (60 mg./kg.) were measured. Details of the technique are given in a previous publication (Graham and Lewis, 1953).

3. *Mode of Action.*—Five pairs of cats were taken and the larger animal of each pair was injected intravenously with 20 mg./kg. of dibenamine hydrochloride. This cat (the donor) was anaesthetized with ether and chloralose (60 mg./kg.) 18–24 hours later, and the response of the blood pressure to adrenaline (4 μg./kg.) recorded. The recipient cat was similarly anaesthetized and the left carotid artery of the donor connected to the cephalic end of the right carotid artery of the recipient; the peripheral end of the femoral vein (or occasionally of the femoral artery) of the recipient was connected to the central end of the femoral vein of the donor. Cross-circulation was maintained during 1–2 hours with the aid of heparin. The cats were then separated and the response of each to injected adrenaline determined.

In these and in 10 other cats, dibenamine hydrochloride (20 mg./kg.) was injected intravenously and the response of the blood pressure to repeated injections of adrenaline (4 μg./kg.) determined after periods of 24, 48, 72, and 96 hours. Adrenaline hydrochloride in a dose of 10 mg./kg. was injected in some of these cats. In others, sodium thiosulphate (1 g./kg.) was injected over a period of 5–10 minutes, followed by continuous infusion from an automatic syringe of 0.3 g./kg./hr. Regular injections of 4 μg./kg. of adrenaline were maintained for periods up to 12 hours. In some cats both procedures were followed. Six cats were injected with J11 (10 mg./kg.) and similarly tested at varying periods up to 48 hours.

4. *Specificity. Blood Pressure.*—The responses of the blood pressure to adrenaline (4 μg./kg.), histamine (2 μg./kg.), acetylcholine (1 μg./kg.), and posterior pituitary extract (0.1 unit/kg.) were demonstrated in cats anaesthetized with chloralose (60 mg./kg.) and the effect of several doses of each haloethylamine determined by the method previously described (Graham and Lewis, 1953). The compounds used were: (*a*) the four N-α-naphthylmethyl-N-ethyl-2-haloethylamines in which the halogen was Cl (J10), Br (J11), I (J12), and F (J13); and (*b*) four N-naphthylmethyl-N-alkyl-bromoethylamines, viz., α-naphthylmethyl derivatives in which the alkyl group was ethyl (J11) or methyl (J21) and β-naphthylmethyl derivatives in which the alkyl group was ethyl (J17) or methyl (J26). An interval of one hour was allowed to elapse between injections of posterior pituitary in order to avoid tachyphylaxis.

Uterus.—Rabbit uterus was mounted in a 10-ml. modified Magnus bath in Locke's solution, oxygenated with O_2+5% CO_2 at 37° C. and *p*H 7.3. Standard responses were obtained to adrenaline (1.0–100 μg./100 ml.) and to posterior pituitary extract (0.05–0.5 unit/100 ml.), alone and in the presence of the haloethylamines (0.1 μg.–10 mg./100 ml.). The spasm-producing agent was added at intervals of 10–15 minutes and allowed to act for one minute before washing. At least four equal successive responses were obtained before a suitable dose of inhibitor was added and allowed to act for three minutes. The degree of inhibition was found for several doses of haloethylamine and the percentage inhibition plotted against the logarithm of the dose. The dose of inhibitor causing 50% inhibition was estimated from the line thus obtained.

Guinea-pig Ileum.—The spasmolytic activity of these compounds was investigated on strips of terminal guinea-pig ileum mounted in Tyrode's solution at 37° C. and *p*H 7.3. The effects of barium chloride (2.5–5.0 mg./100 ml.), acetylcholine (1.0–2.0 μg./100 ml.), and histamine (1.0–2.0 μg./100 ml.) were found alone and in the presence of atropine (0.01–500 μg./100 ml.), papaverine (0.5–5.0 mg./100 ml.), mepyramine (0.01 μg.–5.0 mg./100 ml.) and the haloethylamines (0.05 μg.–3.0 mg./100 ml.). The dose of each haloethylamine causing 50% inhibition of the spasm caused by each of the three agents was estimated and compared with that for atropine, papaverine, and mepyramine.

Results

Aged solutions of haloethylamines J10, J11, J12, and J13—viz., α-$C_{10}H_7CH_2N(Et)CH_2CH_2X$, where X=Cl (J10), Br (J11), I (J12), and F (J13), as free base, were tested by the methods 1 and 2 above.

The fluoro compound did not form any ethyleneiminium ion (Chapman, James, Graham, and Lewis, 1952). No uptake of thiosulphate or pharmacological activity was recorded. J11 and J12 cyclized very rapidly, over 98% of the theoretical maximum transformation occurring within 2 minutes. The amount of residual haloethylamine (2%) was small enough to be disregarded. With J10, the formation of the intermediate was slow, (maximum in 40 minutes) and was incomplete (maximum observed 35% of the theoretical). The residual haloethylamine was thus considerable and unless removed from solution might be active *in vivo*. Before assaying the solution of J10 the sample was washed twice with an equal volume of ether in a separating funnel immediately before injection. This additional procedure removed all uncyclized haloethylamine, leaving only the cyclic ethyleneiminium ion.

The results of the experiments are shown in Table I, where the concentrations of ethyleneiminium ions, the antiadrenaline activity and the antihistamine activity are given for solutions of various ages. In Figs. 1, 2 and 3, the curves are shown relating the percentage of ion to time, —that is, age of solution—for J10, J11, J12 respectively. The points on the three figures represent the percentage of antiadrenaline and antihistamine activity plotted against age of solution. These points fit well to the curves which represent the concentration of ethyleneiminium ion.

Table I

THE CHEMICAL REACTIVITY AND PHARMACOLOGICAL ACTIVITY OF "AGED" SOLUTIONS OF SOME HALOETHYLAMINES

Compound	Age of Solution (min.)	Antiadrenaline Activity (% of Theoretical Maximum)	Antihistamine Activity (% of Theoretical Maximum)	g. Ion Produced/Mole Reactant %
J10	5	11		6
	7·5	14	16	17
	10	20		20
	30	42	34	35
	50	42		34
	150		15	22
	180	21		19
	270		10	14
J11	0	100	100	98
	30		73	74
	60	72	60	64
	90	54		57
	180	32	40	40
	270	30		28
	360	23	20	19
	540	5		10
J12	0	100	100	98
	45	89		80
	90	70	59	66
	180	43		50
	240	38	42	40
	330	29		33
	360		29	31
	480	23		25

The course of deterioration of pharmacological activity was followed in 0.02 M solutions of J10, J11, and J12 in 3:2 (v/v) acetone-water. The table shows the percentage of theoretical maximum antiadrenaline and antihistamine activity in "aged" solutions. In the last column of the table is shown the amount of substituted ethyleneiminium ion, expressed as the percentage of the theoretical maximum. It will be noted that there is good correlation between antiadrenaline activity (column 2), antihistamine activity (column 3) and amount of ethyleneiminium ion (column 4).

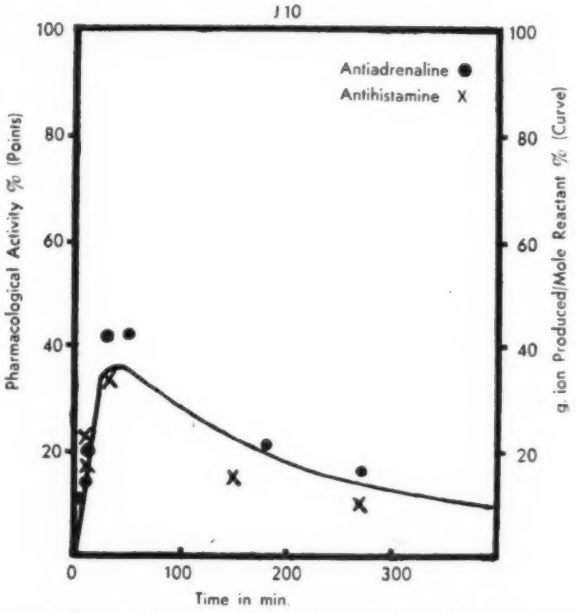

FIG. 1.—To show the relationship between pharmacological activity (antiadrenaline action and antihistamine action) and the amount of ethyleneiminium ion in an ageing solution containing 0.02 M. J10 in 3: 2, v/v, acetone-water. The curve indicates the amount of ethyleneiminium ion present expressed as a percentage of the theoretical maximum of g. ion produced per mole of J10. The antiadrenaline activity was assayed against the pressor responses to adrenaline in spinal cats, and the antihistamine activity against the depressor response to histamine in cats anaesthetized with chloralose.

The activity possessed by freshly prepared aqueous-acetone solutions of J11 or J12 was designated as 100%. This could not be done with J10, so solutions were assayed 40 minutes after they had been made—i.e., when the concentration of ethyleneiminium ion was chemically estimated to be 35% of the theoretical maximum; the results were adjusted accordingly.

It must be emphasized that the chemical estimations were made on solutions very different from blood or other body fluids. It cannot be assumed that the reactions: haloethylamine→ethyleneiminium ion→hydrolysis product, will follow the same time course when a haloethylamine is injected into an animal as they do in artificial systems; but the ageing solution provides samples

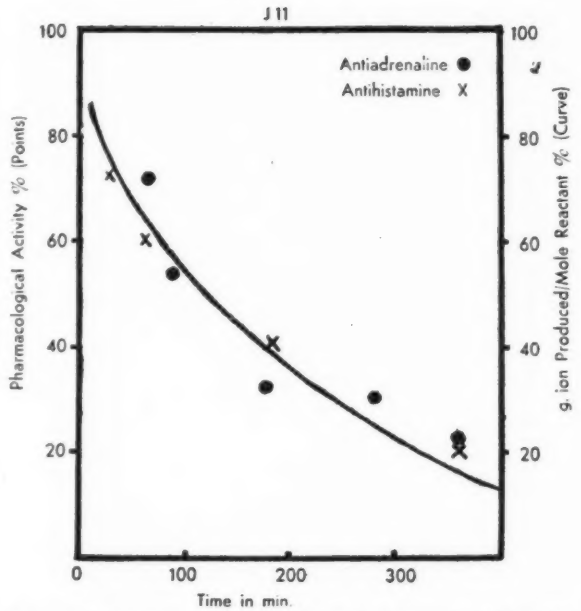

FIG. 2.—To show the relationship between pharmacological activity (antiadrenaline action and antihistamine action) and the amount of ethyleneiminium ion in an ageing solution containing 0.02 M. J11 in 3 : 2, v/v, acetone-water. As in Fig. 1, the curve represents percentage of ion and the points represent percentage of pharmacological activity. With this compound, as with J10, both sets of points fit the curve.

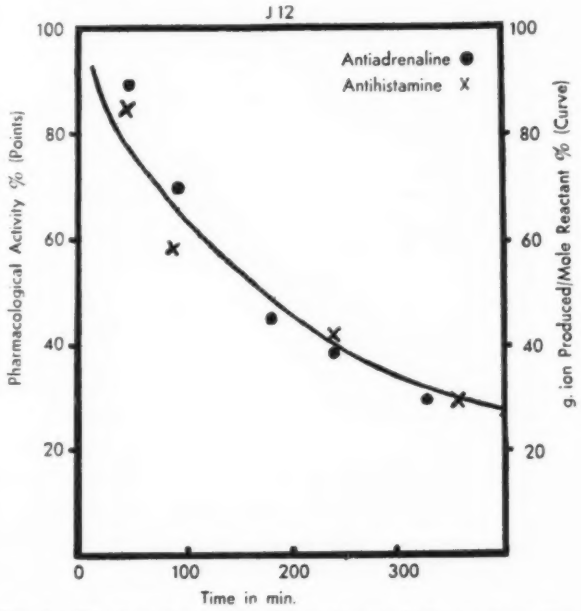

FIG. 3.—To show the results of experiments similar to those illustrated in Figs. 1 and 2, using J12. Again both sets of points fit the curve.

of the ethyleneiminium ion of different, but known, concentrations, and the potencies of these samples were estimated biologically, so that the potency could be correlated with the ethyleneiminium ion content. Thus in Fig. 2, when the solution was one hour old, it contained 64% of the compound J11 present as ethyleneiminium ion ; at this time the solution had 72% of its original antiadrenaline activity and 60% of its original antihistamine activity. After three hours, the solution contained 40% of ethyleneiminium ion, 32% of its antiadrenaline activity and 40% of its antihistamine activity. Similarly, the other points lie on the curve, as also do the points in Figs. 1 and 3, for J10 and J12 respectively. The antiadrenaline and antihistamine activities are thus directly proportional to the amount of ethyleneiminium ion present in solution.

The results of the experiments on the responses of cat blood pressure to adrenaline, histamine, acetylcholine and posterior pituitary extract are shown in Table II. The pressor response to adrenaline was reversed by all the compounds in the table except J13 (a fluoro derivative). The depressor response to histamine was reduced by all the compounds, but J13 only caused slight reduction in the response after a very large dose. The pressor response to posterior pituitary extract was not reduced by any of the compounds, and the depressor responses to acetylcholine were only slightly diminished by some of the haloethylamines.

TABLE II

THE ACTION OF THE HALOETHYLAMINES ON THE RESPONSE OF BLOOD PRESSURE TO ADRENALINE, HISTAMINE, ACETYLCHOLINE AND PITUITARY (POSTERIOR LOBE) EXTRACT. (MEAN OF 3 READINGS ON 3 ANIMALS)

Compound	Dose (mg./kg.)	Lowering of B.P. (% of Initial)	% Reduction in Responses of B.P. after Admin. of Haloethylamines			
			Adrenaline (4 μg./kg.)	Histamine (2 μg./kg.)	Acetylcholine (1 μg./kg.)	Post. Pit. (0·1 unit kg.)
J10	0·05	0	5	24	3	5
	0·5	10	R	85	8	0
J11	0·05	3	10	35	10	0
	0·5	9	R	84	16	0
J12	0·05	0	18	40	0	0
	0·5	0	R	74	0	0
J13	0·05	0	P	0	4	0
	0·5	0	P	4	0	0
	10·0	0	P	37	0	0
J17	0·05	7	10	2	0	0
	0·5	12	R	62	0	0
J21	0·05	2	P	2	0	0
	0·5	8	45	27	4	0
	1·0	14	R	43	9	0
J26	0·05	0	P	10	2	0
	0·5	0	5	20	0	0
	3·0	10	R	50	0	0

P = Potentiation of pressor response to adrenaline 12–200%.
R = Reversal of pressor response to adrenaline.

The table shows the effect of the haloethylamines on the responses of cat blood pressure (anaesthetic; chloralose 60 mg./kg.) to adrenaline 4 μg./kg., histamine 2 μg./kg., acetylcholine 1 μg./kg., and pituitary (posterior lobe) extract 0.1 unit/kg. All the active compounds show great specificity in inhibiting responses to adrenaline and histamine.

In the cross-circulation experiments it was found that 18–24 hours after injection of 20 mg./kg. of dibenamine the response of the donor cat to 4 μg./kg. of adrenaline was reversed. There was insufficient dibenamine in the circulation of the donor to effect an antagonism to adrenaline in the recipient cat. In the donor cats and others similarly injected with dibenamine or J11 the "blockade was absolute," a reversal of the usual pressor response to adrenaline persisting with doses of 10 mg./kg. Only towards the end of the fourth day (dibenamine) or the second day (J11) did a competitive phase precede the disappearance of the effect of the antagonist.

At no time (2 hours to 4 days after dibenamine, 1–24 hours after J11) did injection or infusion, or both, of thiosulphate modify the effect or duration of action of dibenamine or J11.

On every such occasion tested, the effect of massive doses of adrenaline (1–10 mg./kg.) was to produce a profound fall in blood pressure. After the pressure had returned to its previous level, further injections of 4 μg./kg. produced either no response or a small pressor response associated with cardiac stimulation. This phase lasted about 30 minutes, was followed by renewal of the reversal, and the cycle could be repeated several times by injection of the large dose of adrenaline. Injection or infusion of thiosulphate after the adrenaline did not modify this phenomenon, which was probably due to temporary exhaustion of the depressor mechanism by large doses of adrenaline.

The spasmolytic action of the haloethylamines was examined on guinea-pig ileum. The results are presented in Table III, where the potencies are expressed as the dose causing a 50% reduction in the response to spasmogenic agents. Comparisons are made with atropine for antiacetylcholine action, with mepyramine for antihistamine action and with papaverine. It can be seen from Table III that the haloethylamines are essentially specific antihistamines, whilst exerting some antagonism of the responses to acetylcholine.

TABLE III

THE ACTION OF THE HALOETHYLAMINES ON THE RESPONSES OF GUINEA-PIG ILEUM TO HISTAMINE, ACETYLCHOLINE AND BARIUM CHLORIDE

Compound	Dose of Compound in μg./100 ml. Causing 50% Inhibition of Response to:		
	$BaCl_2$ (2·5–5 mg./100 ml.)	Acetylcholine (1–2 μg./100 ml.)	Histamine (1–2 μg./100 ml.)
Atropine ..	120	0·1	100
Papaverine ..	2,100	2,300	2,000
Mepyramine ..	1,250	2,000	0·14
J10	>2,000	100	0·19
J11	>2,000	75	0·35
J12	>2,000	150	0·33
J13	>2,000	>5,000	700
J17	>2,000	300	0·4
J21	>2,000	200	10·0
J26	>2,000	300	15·0

Guinea-pig ileum mounted in a modified Magnus bath in Tyrode's solution, at 37° C., *p*H 7.3, oxygenated with O_2 + 5% CO_2. The table shows the dose of compound in μg./100 ml. causing 50% inhibition of the responses to barium chloride 2.5–5 mg./100 ml., acetylcholine 1–2 μg./100 ml., and histamine 1–2 μg./100 ml.

TABLE IV

THE EFFECT OF THE HALOETHYLAMINES ON THE CONTRACTIONS OF ISOLATED RABBIT UTERUS TO ADRENALINE AND PITUITARY (POSTERIOR LOBE) EXTRACT

Compound	Dose of Compound Producing 50% Inhibition of Response to:	
	Adrenaline (1–100 μg./100 ml.)	Post. Pituitary (0·05–0·5 unit/100 ml.)
J10	0·8	7,000
J11	0·7	5,000
J12	1·5	10,000
J13	700	7,000
J17	1·6	5,000
J21	30·0	6,000
J26	50·0	10,000

Isolated rabbit uterus mounted in Locke's solution in a modified Magnus bath at *p*H 7.3, and 37° C. oxygenated with 5% CO_2 in O_2.

Standard additions of stimulating agents were made at 10–15 min. intervals and repeated after addition of a haloethylamine. The values for 50% inhibition were obtained from a plot of percentage inhibition against the logarithm of dose of the haloethylamine.

The specificity of the antagonism of the responses to adrenaline is clearly shown.

The results of the experiments on rabbit uterus are given in Table IV. Here again the haloethylamines are shown to be specific—this time as anti-adrenaline agents.

DISCUSSION

A quantitative relationship between the concentration of the ethyleneiminium ion formed by J10, J11, and J12 and the specific pharmacological activity of these compounds has been observed. As the haloethylamines are known to form the ethyleneiminium ion *in vitro*, and the pharmacological activity of the haloethylamines is known to vary directly with the concentration of ethyleneiminium ion, it may be deduced that such cyclic ions are formed when the haloethylamines are injected *in vivo* and that they are the active species. Thus the correctness of Nickerson's supposition, that the pharmacological activity of these compounds is due to the formation of active intermediates, has been rendered more probable by experimental evidence.

A serious objection to this view has been raised by Axelrod, Aronow and Brodie (1952), who examined the distribution and metabolic fate of dibenamine in the dog. According to the present theory, dibenamine would be cyclized and sub-

sequently converted to dibenamine alcohol (N-dibenzylaminoethanol). These authors were unable to detect the latter substance in the urine after administration of dibenamine. In order to determine whether or not the alcohol itself was metabolized, they injected dibenamine alcohol 30 mg./kg. into two dogs and found the proportion excreted in the urine to be 10–15%. They concluded from this that dibenamine alcohol was *not* metabolized, and used the conclusion as evidence that the alcohol was not formed from dibenamine in the body, suggesting that the antiadrenaline action was not due to the ethyleneiminium ion. It seems reasonable to ask, however, how the 85–90% of the alcohol was lost if not as a result of metabolic activity.

Axelrod *et al.* further showed that five minutes after injection of dibenamine, 60% of the latter is lost (see Table 5 of their paper). If this were converted to the corresponding alcohol, then 90% of the latter would "disappear" leaving approximately 6% of the original dibenamine to be present in the urine as alcohol. As the stated error for the method of estimation is ±5%, the argument against this mechanism occurring appears to be weak. If the 60% of dibenamine was not immediately metabolized but was held in combination by cell constituents, it would be expected that a slow release of the amine from combination would give rise to a slow formation of alcohol which would consequently be metabolized before reaching the urine.

If it be accepted that the presence of the ethyleneiminium ion is essential for the specific effect of the haloethylamines in antagonizing the actions of adrenaline and histamine, it is possible to consider the actions of these compounds in more detail. It has been shown that the compounds vary in their antiadrenaline and antihistamine potency. If the activity depends upon the ethyleneiminium ion formed, it would be logical to conclude that the structural features present in J21 and J26 as compared with those of J11 and J17 reduce the ability of the two former compounds to form the cyclic intermediate.

These compounds at higher concentrations reduce the response of guinea-pig ileum to acetylcholine. Their potencies in this respect do not bear the same relationship to one another as do their antihistamine potencies. The fact that a very high concentration of the fluoroethylamine J13 is needed indicates that activity resides in the ion rather than in the molecule. The concentrations required to reduce the responses of guinea-pig ileum to barium chloride or of rabbit uterus to pituitary extract are such as to preclude any accurate measurement.

We have observed previously (Graham and Lewis, 1953) that the group of haloethylamines examined exhibited two types of toxic symptoms, stimulation and depression. The former was quick in onset and caused death within 1 to 2 hours, whereas the depression was slower in onset and death occurred 12 to 48 hours after administration. The fluoro derivatives which do not form the cyclic ethyleneiminium ion caused a rapid convulsive death; the chloroethylamines which cyclize less readily than the bromo or iodo derivatives (see Fig. 1) caused some initial stimulation followed by prolonged depression; while the bromo- and iodoethylamines which rapidly and completely cyclize gave rise to a prolonged depression with delayed death. It would appear that the original haloethylamine molecule causes convulsive symptoms leading to a rapid death, while the cyclized ion formed from it causes a state of prostration in which the onset of death is delayed.

Mode of Action

The facts already discovered about the haloethylamines (Nickerson and Nomaguchi, 1948; Graham and Lewis, 1953) suggest that they act by blocking the receptor mechanism in certain smooth muscle cells which respond to adrenaline with a motor response (Ahlquist, 1948), but not in others which relax, or in the heart.

Nickerson and Goodman (1948) suggested that dibenamine reacted irreversibly with some cellular constituent which is essential for reaction to adrenaline stimulation. They reached this conclusion from the indirect evidence that the action of the drug may persist for several days beyond the period that an effective concentration can be demonstrated in the body. The test consisted of cross-circulation in dogs, the first dog having received a large dose of dibenamine. The time was noted when cross-circulation failed to produce antiadrenaline action in a second dog. Our experiments confirm these results in cats.

Axelrod *et al.* showed that some 20% of dibenamine was localized in fat two hours after injection into a dog, and that formation of dibenzylamine was also important (24% unchanged dibenamine recovered from all tissues of a dog five minutes after injection, and another 16% as dibenzylamine; this leaves 60% unaccounted for). The prolonged action of dibenamine is attributed by these authors to gradual release from the fat depôt, so that the drug is present though chemically undetectable in blood the whole of the time

that its action is evident. After injection of 47 mg./kg. in a dog the amount of dibenamine absorbed in the fat was estimated to be 26 mg./kg. at two hours and 24 mg./kg. at 27 hours.

We have carried out a series of experiments in order to test whether the prolonged action of dibenamine was due to the release of the compound from fat depôts. It has been shown that administration of thiosulphate before injection of the antiadrenaline compound prevents the antagonism of the pressor response to adrenaline from developing. The mechanism is by chemical interaction between the thiosulphate and the antagonist. It has also been observed that administration of thiosulphate after the antagonism has been established does not affect its course. We have extended these studies, having administered sodium thiosulphate by injection and by continuous infusion into cats injected intravenously with 20 mg./kg. of dibenamine 18–24 hours previously. After this time the dibenamine in the circulation is not detectable biologically by cross-circulation, so that the prolonged action is due either to release from fat depôts (Axelrod *et al.*) or to a stable dibenamine-receptor complex (Nickerson). After treatment with thiosulphate any dibenamine in the circulation would be neutralized so that release from fat depôts would be ineffective. In such circumstances, the duration of dibenamine antagonism would be brief. This is not so (up to 12 hours). Nevertheless a relationship exists between the dose of antagonist administered and the duration of the antagonism ; thus, with J11, 1 mg./kg. antagonizes adrenaline for 6–8 hours, 10 mg./kg. for approximately 48 hours ; with dibenamine, 5 mg./kg. lasts for approximately 36 hours, 20 mg./kg. for four days. Thus the absorption of dibenamine into the fat, although it may play some part, is not the limiting factor in the prolonged action of the drug.

Further proof of a chemical reaction with tissue constituents has been afforded by the observations of Ferguson and Wescoe (1950), who examined the action of N-dimethyl-2-chloro-2-phenylethylamine ($C_6H_5CHClCH_2NMe_2$) and its intermediate products. Two important observations were made, namely, that the duration of the antiadrenaline action *in vivo* was greater than the time in which the ion is hydrolysed to the inactive alcohol in serum *in vitro*, and that thiosulphate, which reacts with the ethyleneiminium ion and prevents its pharmacological activity, does not affect the degree of inhibition once established.

We have also obtained indirect evidence in support of this hypothesis in that the action of the haloethylamines *in vitro* is not removed by washing, while that *in vivo*, although durable, is reversible. Thus, a reaction, with some essential constituent that can eventually be replenished *in vivo* and cannot be reversed by washing a tissue *in vitro*, is indicated.

Graham and Lewis (1951, 1953) have compared the antihistamine and antiadrenaline actions of the haloalkylamines. While these activities have many features in common there are certain differences. Both the antagonisms are exerted within a very short time of administration *in vivo*, but whereas that to adrenaline continues for many hours the antihistamine action is of comparatively short duration.

In very small doses the haloethylamines potentiate the pressor response to adrenaline, probably owing to the known inhibition of amine oxidase ; but there is no potentiation of histamine, nor is there any effect on histaminase. The action is due to purely structural factors, as the fluoroethylamines which do not form active intermediates cause inhibition of monoamine oxidase and markedly potentiate the pressor response to adrenaline (see Table II). In slightly larger doses active haloethylamines exert a competitive inhibition of the responses to adrenaline. This phase may be explained by assuming that below the limiting concentration for non-competitive antagonism, some of the receptors would not be occupied by antagonist and would thus be still available to adrenaline. Alternatively, there is a limiting concentration of the antagonist below which it will not attach itself to the receptor sufficiently strongly to overcome the active factors which remove and destroy it. In higher doses the pressor response to adrenaline is inhibited in a non-competitive manner, while the depressor response to histamine is always inhibited competitively at all dose levels, —again indicating a difference in the nature of the interaction of the antagonist and the two types of receptor.

Summary

1. The examination of a group of haloethylamines reported in a previous publication has been extended to a study of their mechanism of action.

2. It is more than probable that the antiadrenaline and antihistamine actions of the haloethylamines are due to the formation of cyclic ethyleneiminium ions.

3. The actions of the compounds have been explained in the light of these findings. The specific antiadrenaline and antihistamine actions,

as well as a weak antiacetylcholine action, are exerted by the cyclized ion, the non-specific actions by the molecule.

4. Their mode of action as antiadrenaline agents is possibly chemical combination with the tissue receptors, but as antihistamines they may act by forming a less stable complex with some constituent of the effector mechanism.

5. No evidence was obtained in support of the view that continuing release of haloethylamines from fat depôts is the limiting factor in the prolonged action of these compounds.

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THE INHIBITION OF CHOLINESTERASES BY ANTAGONISTS OF ACETYLCHOLINE AND HISTAMINE

BY

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A well-recognized group of drugs has the property of inhibiting cholinesterases. The present paper is concerned mainly with the inhibition of cholinesterases by drugs which do not primarily fall into this group but which appear to influence the action of acetylcholine.

Some 50 compounds were studied in respect of their ability to inhibit the true and pseudo cholinesterases, as typified by homogenates of rat brain and rat intestinal mucosa respectively (Ord and Thompson, 1950).

A summary of this work was communicated to the symposium on anticholinesterases, organized jointly by the British Pharmacological Society and the Society of Chemical Industry (Fine Chemicals Division), and held at the Wellcome Research Institution on March 27 and 28, 1953.

METHODS

The cholinesterase activity was estimated by a modification of Ammon's (1933) method. The details are as follows:

Technique.—The substrate and the inhibitor were mixed in the well of the Warburg flask and the enzyme was placed in the side-arm. This served to minimize the delay in the attainment of maximum enzymic activity noticed by Goldstein (1944). After tipping, at least five minutes were allowed for temperature re-equilibration before the first reading was taken. The intervals between readings were three minutes for the brain enzyme and six minutes for the intestinal mucosa enzyme. The results were calculated by the method of Aldridge, Berry, and Davies (1949).

Enzyme Preparations.—Male albino rats of 250–350 g. were used throughout. The animals were killed by fracture of the neck. The brain was dissected free, washed in saline, and homogenized immediately in a high-speed homogenizer in Krebs-Henseleit saline (18.5 ml. saline per g. wet weight). The small intestine was removed, washed through with saline, and split longitudinally; the mucosa was scraped off with a spatula and suspended in 20 ml. Krebs-Henseleit saline. This was homogenized and then diluted as required, 1 in 4–6, according to the activity of the preparation.

Using 0.5 ml. per flask, the output from these preparations was 200–300 μl. CO_2 per hour.

Substrates.—Acetylcholine chloride (Roche) was used in all experiments. Solutions were made up in distilled water and brought to *p*H 4 to minimize hydrolysis before use. A final concentration of 0.0025 M was employed with the brain preparation, and of 0.0625 M with the mucosa; each of these concentrations is close to the optimum value. When comparing the activities of true and pseudo cholinesterases against the physiological substrate most workers have used the same substrate concentration —usually 0.015 M—with both enzymes. Augustinsson (1949) has, however, criticized the use of a substrate concentration above the optimum with the true cholinesterase; our experience fully supports his criticism (Todrick, Fellowes, and Rutland, 1951).

Drugs.—These were dissolved in Krebs-Henseleit saline in concentrations 50% higher than those finally required. The addition of 2.0 ml. to the remaining reagents gave the correct concentration. Some drugs were insufficiently soluble to produce the desired 50% inhibition. Where extrapolation could reasonably be carried out, an approximate result has been quoted and indicated as such.

Controls.—In the earlier experiments, the enzyme blank was determined both in the absence of inhibitor and in the presence of the highest inhibitor concentration used; the value for the substrate blank was taken from a previous paper (Todrick *et al.*, 1951). In later experiments the first of the two controls, for which the value lay between 0 and 16 mm.^{3}/hr., was omitted, since it formed part of the second control. The flasks thus freed were used for a direct estimation of the substrate blank. This procedure is safer for the measurement of enzyme activity, but makes it rather less easy to recognize the effect of the homogenate on the drug when the effect is small.

Calculation of Results.—The inhibitory potency of each drug was determined at four or five concentrations, the total number of estimations on the inhibited enzyme being not less than twelve. From these results, the percentage inhibition-log concentration curve was drawn by hand, and the concentration causing

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50% inhibition was read off. This was converted to its negative logarithm.

The ratio of the 50% inhibition concentrations for the two enzymes has been calculated in the form [I]50 brain/[I]50 mucosa, subsequently abbreviated to " B/M ratio." This index is a measure of the specificity of the drug for one enzyme or the other. Since a high [I]50 denotes a low specificity, a B/M ratio >1 indicates preferential inhibition of the pseudo cholinesterase.

Results

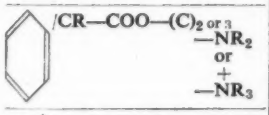
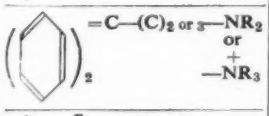
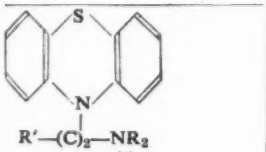
Forty-six substances have been examined for inhibitory action on the two cholinesterases. The drugs can be classified as:

1. Neuromuscular blocking agents.
2. Antimuscarinic agents.
3. Drugs effective in Parkinsonism.
4. Antihistaminic agents.
5. Anticholinesterases.
6. Miscellaneous substances.

The chemical formulae of the drugs in the first three classes are derived from one of the four basic structures shown in Table I.

TABLE I

BASIC CHEMICAL STRUCTURES FOUND AMONG NEUROMUSCULAR BLOCKING AND ANTI-MUSCARINIC DRUGS AND DRUGS EFFECTIVE IN PARKINSONISM

Basic Chemical Structure	Notes	Structure Code Used in Tables II to IV
$R_3\overset{+}{N}-(C)_{9-11}-\overset{+}{N}R_3$	Some of the C atoms may be part of ring systems; one or two can be replaced by ether linkages	A
	R is often another phenyl group; the phenyl groups can be replaced by other cyclic structures	B
	The phenyl groups can be replaced by other cyclic structures	C
	R′ is either H or a methyl group	D

The individual compounds examined in each class are listed in alphabetical order in Tables II–VII respectively, together with the code letters indicating the basic structure (Tables II–IV only), and the experimental results.

Each drug has been classified in accordance with its best recognized action. The distinction between antimuscarinic substances and drugs effective in Parkinsonism rests not so much on the lack of antimuscarinic properties in the latter as upon their greater effectiveness in this syndrome. It is possible that this effect is merely a manifestation of antimuscarinic action at a central rather than a peripheral site. Alternatively, drugs effective in Parkinsonism may possess additional actions, probably related to their antagonism to the central effects of nicotine (Bovet and Longo, 1951).

Many of the drugs have relatively powerful secondary actions and one, promethazine hydrochloride (" Phenergan "), seems to possess three major actions—antihistaminic, antimuscarinic, and effectiveness in Parkinsonism. Account must be taken of this in considering the experimental results.

The Effect of Cholinesterases on Drugs.—Bovet-Nitti (1949) showed that succinylcholine and other synthetic paralysing drugs were hydrolysed by cholinesterases; it was therefore considered desirable to determine whether, and to what extent, this occurred with the drugs and enzyme preparations used in this work. Hydrolysis would introduce two errors: it would appear as an increase in the enzyme activity and it would cause a fall in the inhibitor concentration.

It was found that three compounds were acted upon by the enzyme preparations, with the formation of acids or CO_2, and, as they were alkamine esters (atropine, lachesine, and IS337), it seemed probable that the cholinesterase was responsible. The hydrolysis only occurred at a measurable rate at very high concentrations (*ca.* 10^{-2}M), and it could be shown, for example with lachesine, that the percentage destruction of the inhibitor did not amount to more than 2% in 30 min. at 38° C.; thus the effect on the inhibitor concentration during the experiment was negligible.

It should be made clear, however, that the hydrolysis of drugs by the enzymes has not been measured at concentrations higher than are necessary to produce nearly complete inhibition. Nevertheless, it would not be expected that a further increase in drug concentration beyond this point would materially increase its hydrolysis rate.

The Inhibition of Cholinesterases by Drugs.—Tables II, III, and IV give the *p*I 50 values and B/M ratios for the neuromuscular blocking and antimuscarinic drugs, and for those effective in Parkinsonism. The compounds are arranged in alphabetical order.

TABLE II

THE INHIBITION OF CHOLINESTERASES BY NEUROMUSCULAR BLOCKING AGENTS

Compound	Structure Type (See Table I)	*p*I 50		B/M Ratio	Pharmacological Properties (References)
		Rat Brain Cholinesterase	Rat Intestinal Mucosa Cholinesterase		
Decamethylene $\alpha:\omega$-bis-6: 7-dimethoxy-1: 2: 3: 4-tetrahydro*iso*quinolinium methiodide (" No. 14," A and H)	A	5·10	4·40	0·19	Taylor and Collier, 1950
Gallamine triethiodide (" Flaxedil," M and B)		2·40	3·05	4·2	Bovet, Depierre, Courvoisier, and de Lestrange, 1949
Bis-(2-diethylaminoethyl) phthalate diethiodide (" IS 302 ")	AB	2·35	3·60	18	Bovet, Bovet-Nitti, Guarino, Longo, and Marrotta, 1949
Bis-(2-diethylaminoethyl) *iso*phthalate, diethiodide (" IS 337 ")	AB	5·35	1·90	0·00035	Bovet, Bovet-Nitti, *et al.*, 1949
8: 8′-(pentamethylenedioxy) bis [1-ethyl-quinolinium iodide] (" RP 3381 ")	A	7·20	4·45	0·0019	Bovet, Courvoisier, Ducrot, and Horclois, 1949
Pentamethylene bis-oxy-*o*-phenylene bis-[trimethylammonium iodide] (" RP 3565 ")	A	5·05	2·80	0·0057	Bovet, Courvoisier, *et al.*, 1949
(+)-Tubocurarine	A	3·30	2·25	0·087	—

TABLE III

THE INHIBITION OF CHOLINESTERASES BY ANTIMUSCARINIC COMPOUNDS

Compound	Structure Type (See Table I)	*p*I 50		B/M Ratio	Pharmacological Properties (References)
		Rat Brain Cholinesterase	Rat Intestinal Mucosa Cholinesterase		
Atropine sulphate	B	1·50	3·20	51	—
Atropine methyl nitrate	B	1·70	3·45	57	—
3: 3-Diphenylpropan-3-ol diethylamine methiodide (" 186C47," Wellcome)	C	2·35	3·20	7·4	White, Green, and Hudson, 1951
Hyoscine hydrobromide	B	1·80	2·20	2·3	—
2′-Diethylaminoethyl-2: 3: 3-triphenyl propionate hydrochloride (" J 4," T. and H. Smith)	B	1–2	3·25	>18	—
N-2-(Diphenylmethylthio)-ethyl piperidine hydrochloride (" J 7," T. and H. Smith)	B	<2·65	*ca.* 3·65	>10	—
Lachesine hydrochloride	B	1·80	2·65	7·0	—
3-N-Piperidino-1: 1-di(2′-thienyl) propan-1-ol (" 5C48," Wellcome)	C	2·35	3·35	10	Adamson and Green, 1950
1-Piperidino-3: 3-diphenyl propan-3-olamine (" 369C46," Wellcome)	C	1–2	3·25	>18	White, Green, and Hudson, 1951
2-Diethylaminoethyl diphenylacetate hydrochloride (" Trasentin," Ciba)	B	2·35	3·00	4·5	Graham and Lazarus, 1940
2-Diethylaminoethyl-α-phenyl cyclohexyl acetate hydrochloride (" Trasentin 6H," Ciba)	B	<2	3·60	>40	Graham and Lazarus, 1940

TABLE IV

THE INHIBITION OF CHOLINESTERASES BY DRUGS EFFECTIVE IN PARKINSONISM

Compound	Structure Type (See Table I)	*p*I 50		B M Ratio	Pharmacological Properties (References)
		Rat Brain Cholinesterase	Rat Intestinal Mucosa Cholinesterase		
1-Cyclohexyl-1-phenyl-3 (1-piperidyl) propan-1-ol (" Artane," Lederle)	C	*ca.* 2	4·00	*ca.* 100	Doshay and Constable, 1949
1-Cyclohexyl-1-phenyl-3-pyrrolidinopropan-1-ol (" Kemadrin," B. W.)	C	2·35	3·95	40	Montuschi, Phillips, Prescott, and Green, 1952
Diethazine hydrochloride (" Diparcol," M. and B.)	D	2·55	5·45	760	Sigwald, Bovet, and Dumont, 1946
Ethopropazine hydrochloride (" Lysivane," M. and B; " RP 3356 ")	D	2·80	6·25	2,800	Durel, 1949; Sigwald, 1949
Ethopropazine methosulphate	D	2·45	5·90	2,800	—
1-Phenylcyclopentane carboxylic acid 2-diethyl aminoethyl ester (" Parpanit " Geigy)	B	2·60	5·30	490	Grünthal, 1946; Schwab and Leigh, 1949
Promethazine hydrochloride (" Phenergan," M. and B.)*	D	2·70	4·45	62	Durel, 1949; Sigwald, 1949
Promethazine methosulphate	D	2·80	4·80	100	—

* Also included in Table V.

It will be seen that, while the individual *p*I 50 values have no particular significance, there appears to be a correlation between the B/M ratio of a drug and its pharmacological action. This is more clearly demonstrated by Fig. 1, which brings out the fact that the neuromuscular blocking agents are, with two exceptions, specific inhibitors of the brain enzyme, whereas the antimuscarinic drugs and those effective in Parkinsonism are specific inhibitors of the mucosa enzyme. For the antimuscarinic drugs the specificity is only moderate, but for those effective against Parkinsonism it is high.

Table V gives the results obtained with histamine and a number of antihistaminic substances. Histamine itself shows no specific inhibitory action, and the same is true of most of the antihistamines tested. Diphenhydramine hydrochloride, promethazine hydrochloride and its isomer " Lergigan," are exceptional in having B/M ratios of 6.0, 62, and 73 respectively.

Three anticholinesterases have been examined (Table VI). Eserine and neostigmine were studied because it was suggested (independently by Professor J. H. Gaddum and Dr. D. Grob) that they would be of special interest since they were specific inhibitors of the pseudo cholinesterase, yet did not possess antimuscarinic activity. However, under the conditions of test used here, eserine has a B/M ratio of 0.064 and neostigmine a ratio of 0.013: both are thus specific inhibitors of the true cholinesterase. In view of this, the inhibition of human serum cholinesterase by these drugs was examined, since this was the enzyme studied by Grob (1949). With eserine there was a large difference in the *p*I 50 for the two pseudo enzymes, the value for human serum being 6.10, and that for rat mucosa 5.25. With neostigmine, the serum enzyme was only twice as sensitive as the mucosa enzyme, and the B/M ratio (0.026) remained considerably less than unity. A major cause of the discrepancy between these and earlier results undoubtedly lies in the substrate concentrations used Grob (1949) used the same substrate concentration for both enzymes, whereas in the present study the concentration used with mucosa and serum was 25 times that used with brain.

The specific anti-true-cholinesterase activity of 62C47 (Burgen, 1948; Austin and Berry, 1953) has been confirmed.

Table VII lists the anticholinesterase activity of a number of miscellaneous drugs. Apart from the two analgesics, none possesses marked specific inhibitory

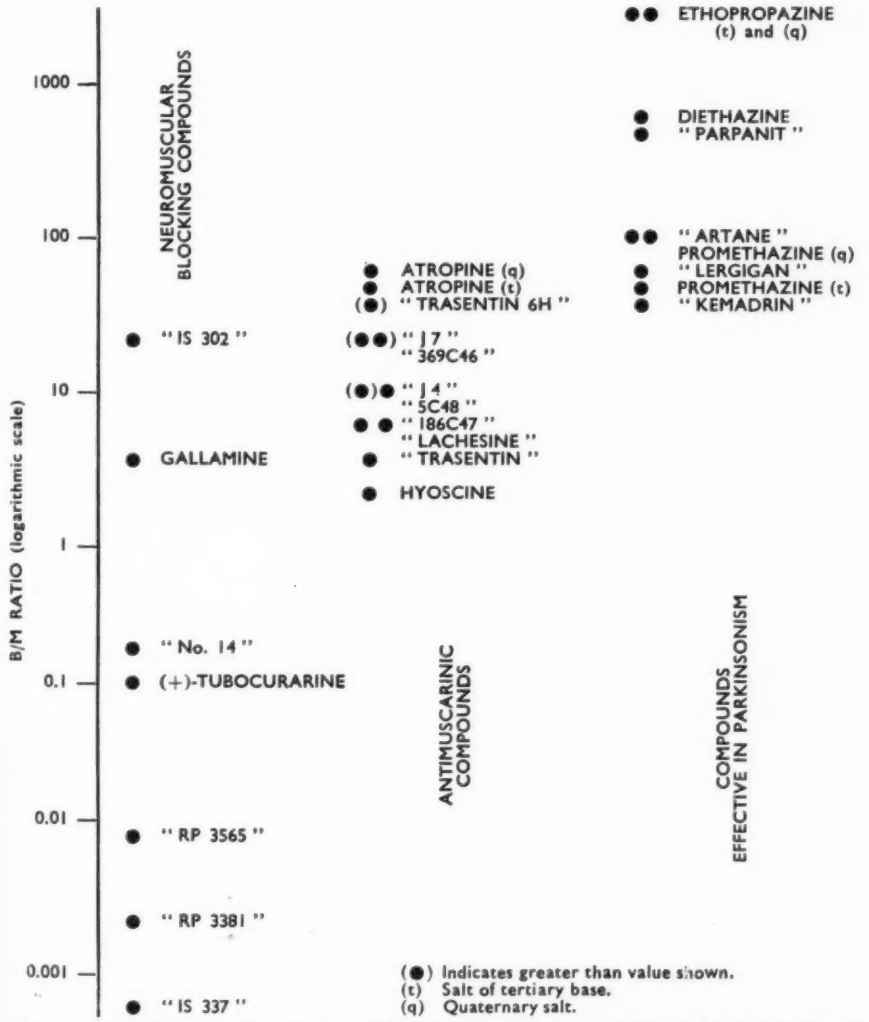


FIG. 1.—The relationship between the specific inhibitory activity of a drug against true or pseudo cholinesterase and its pharmacological action. The B/M ratio is [I]50 brain cholinesterase/[I]50 mucosa cholinesterase. A B/M ratio >1 denotes that the pseudo cholinesterase is preferentially inhibited, a ratio < 1 that true cholinesterase is preferentially inhibited.

TABLE V

THE INHIBITION OF CHOLINESTERASES BY ANTIHISTAMINE DRUGS

Compound	*p*I 50		B/M Ratio	pA_2	
	Rat Brain Cholinesterase	Rat Intestinal Mucosa Cholinesterase		Acetylcholine[1]	Histamine
Antazoline hydrochloride (" Antistin," Ciba)	3·90	4·10	1·5	5·47[1]	7·67[1]
trans-1-(4′Chlorophenyl)-1-(2′pyridyl)-3-pyrrolidinoprop-1-ene maleate (" 405C49," Wellcome)[4]	2·60	2·50	0·7	—	—
10-(1′-Dimethylamino 2′-propyl) phenothiazine hydrochloride (" Lergigan,"[6] AB Recip)	2·55	4·40	73	—	—
Diphenhydramine hydrochloride (" Benadryl," P.D. & Co.)	2·05	2·85	6·0	6·57[2]	8·02[2]
Phenindamine phosphate (" Thephorin," Roche)	2·80	3·20	2·5	—	—
Promethazine hydrochloride[5] (" Phenergan," M. and B.)	2·70	4·45	62	7·62[1]	9·18[1]
Mepyramine maleate (" Anthisan," M. and B.)	3·25	3·05	0·66	4·86[2] 5·03[1]	9·32[1] 9·46[2]
Tripellenamine hydrochloride (" Pyribenzamine," Ciba)	3·70	2·95	0·18	—	—
Histamine	2·15	2·30	1·5	—	—

[1] Figures taken from Reuse (1948). [2] Figures taken from Schild (1947). [3] Adamson, Barrett, Billinghurst, Green, and Jones (1951).
[4] pA_2 for atropine is 8·61 (Schild, 1947). [5] Also included in Table IV. [6] Gernandt and Schmiterlöw (1953).

TABLE VI

THE INHIBITION OF CHOLINESTERASES BY CERTAIN ANTICHOLINESTERASES

Compound	*p*I 50		B/M Ratio	Pharmacological Properties (References)
	Rat Brain Cholinesterase	Rat Intestinal Mucosa Cholinesterase		
Eserine	6·45	5·25	0·064	—
Neostigmine methosulphate	6·70	4·80	0·013	—
1 : 5-Bis-(4′-trimethyl ammoniumphenyl)pentan-3-one diiodide (" 62C47," Wellcome)	7·20	2·45	0·00002	Mogey, 1952

TABLE VII

THE INHIBITION OF CHOLINESTERASES BY MISCELLANEOUS DRUGS

Compound	Pharmacological Action	*p*I 50		B/M Ratio	Pharmacological Properties (References)
		Rat Brain Cholinesterase	Rat Intestinal Mucosa Cholinesterase		
Arecoline	Parasympathetic stimulant	2·40	1·85	0·28	—
3-Diethylamino-1 : 1-di(2′-thienyl) but-1-ene (" 191C49," Wellcome)	Analgesic	3·10	4·10	9·4	Adamson and Green, 1950
3-Dimethylamino-1 : 1-di(2′-thienyl) butane (" 489C49," Wellcome)	Analgesic	2·90	3·90	10	Adamson, Duffin, and Green, 1951
Hexamethonium bromide	Antagonist to decamethonium bromide	1·75	1·75	1·2	Paton and Zaimis, 1949
Lobeline	Carotid sinus stimulant	3·15	3·30	1·5	—
Mephenesin (" Myanesin," B.D.H.)	Spinal cord depressant	—*	—*	—*	Berger and Bradley, 1946
Nicotine	Autonomic ganglion stimulant and depressant	2·75	2·35	0·40	—
Pilocarpine	Parasympathetic stimulant	2·65	1·80	0·15	—

* See text.

action, or indeed much inhibitory action at all. Mephenesin (" Myanesin ") behaves in an interesting manner. Firstly, it is incapable of causing more than 30% inhibition of either enzyme at a concentration as high as 0.03M ; secondly, it even activates the mucosa enzyme slightly over the concentration range 10^{-4}–10^{-2}M, whereas it does not activate the brain enzyme.

DISCUSSION

The experimental data summarized in Fig. 1 suggest that there may be a correlation between the pharmacological action of the compounds examined and their specificity as cholinesterase inhibitors. The majority of the neuromuscular blocking agents appear to be specific inhibitors of the brain or true cholinesterase, whereas anti-

muscarinic compounds and those effective in Parkinsonism tend to be specific inhibitors of the intestinal mucosa or pseudo cholinesterase.

The results obtained with a series of antihistaminic compounds support this idea. Of the eight compounds examined, three—promethazine, its isomer lergigan, and diphenhydramine—preferentially inhibit the pseudo cholinesterase. Promethazine and diphenhydramine are known to possess antimuscarinic activity (cf. the pA_2 values against acetylcholine, determined by Reuse (1948) and Schild (1947) respectively, which are quoted in Table V, col. 5). Lergigan is also said to possess "very strong anti-acetylcholine action" (Gernandt and Schmiterlöw, 1953).

Further evidence favouring the idea of a correlation between pharmacological action and anticholinesterase specificity can be obtained from the published results of other workers.

Blaschko, Chou, and Wajda (1947) found that a series of synthetic esters with atropine-like actions markedly inhibited horse serum cholinesterase at a concentration of 6×10^{-3}M, whereas the cholinesterase of the dog caudate nucleus was affected only slightly or not all all by this concentration.

Paton and Zaimis (1949) showed that the neuromuscular blocking agent dodecamethylene bis-trimethylammonium iodide (C12) is a specific inhibitor of true cholinesterase, the ratio for the 50% inhibition concentrations (human erythrocyte/human plasma) being 0.002; 62C47 (Wellcome), which possesses neuromuscular blocking activity (Mogey, 1952), is also a highly specific inhibitor of the true cholinesterase (Burgen, 1948; Austin and Berry, 1953; see also Table VI).

Acknowledgment must be made of published work on the inhibition of cholinesterases by compounds which I also have examined—such as antazoline and mepyramine (Payot, 1946); diethazine (Gordon, 1948); (+)-tubocurarine (Harris and Harris, 1944); RP3381 and RP3565 (Bovet, Courvoisier, Ducrot, and Horclois, 1949). Some of the published data are qualitative rather than quantitative; nevertheless, when account is taken of the different sources of enzyme and the different substrates and substrate concentrations used, there seems to be no evidence of disagreement between these results and mine.

Whatever the reason for this correlation may be, it should be stressed that the absolute inhibitory activities of many of the compounds are low, and there can be no question of explaining their pharmacological action in terms of their anticholinesterase activity.

The two most obvious exceptions to the apparent correlation between pharmacological action and anticholinesterase specificity are the structurally related compounds gallamine triethiodide and IS302; the former is the pyrogallol triether and the latter the phthalic diester of the same substituted ethanolamine. Though both are stated to possess neuromuscular blocking activity (Bovet, Depierre, Courvoisier, and de Lestrange, 1949; Bovet, Bovet-Nitti, Guarino, Longo, and Marrotta, 1949), they preferentially inhibit the pseudo-cholinesterase, their B/M ratios being 4.2 and 18 respectively.

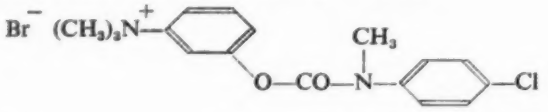
However, Marbury, Artusio, Wescoe, and Riker (1951), in a clinical study of gallamine, have described a vagolytic action which was noticeable before the onset of curare-like symptoms; this is in keeping with the previous finding of Riker and Wescoe (1951) that in cats "the vagal action exceeds the neuromuscular blocking action not only in potency but also in duration." But they also conclude that gallamine "manifests no other atropine-like action."

Hyoscine is another exception, since its B/M ratio 2.3 is the lowest found for any antimuscarinic compound. No explanation for this exception can be offered.

So far, this discussion has centred on the relationship of pharmacological and biochemical properties. It is perhaps worth while to consider shortly the question of chemical structure.

The relationship between pharmacological activity and chemical structure among neuromuscular blocking and atropine-like drugs has been intensively studied (cf. Barlow and Ing, 1948; Kimura, Unna, and Pfeiffer, 1949; Paton and Zaimis, 1953, for neuromuscular blocking drugs; and Gaddum, 1944; Pfeiffer, 1948; Ing, Dawes, and Wajda, 1945, for atropine-like drugs). The evidence suggests that neuromuscular blocking agents are, with the exception of the erythrina alkaloids, all quaternary ammonium salts—or onium salts in general—but that the most potent compounds contain two quaternary N-atoms separated by a relatively long chain, as in decamethonium and gallamine triethiodide, or a bulky ring structure as in tubocurarine. Atropine-like compounds are mostly alkamine esters of acids containing one or two aromatic nuclei, and the basic N-atom (which may be tertiary or quaternary) is usually separated from the ester group by a short chain of 2 or 3 C-atoms; White, Green, and Hudson (1951) have, however, found potent atropine-like compounds of the general structure $Ar_2(C)_3NR_2$ which do not contain an ester group.

If the conclusions drawn in the earlier part of the discussion are correct, true cholinesterase inhibitors should possess the structures associated with the neuromuscular blocking drugs, whereas pseudo cholinesterase inhibitors should possess those of the atropine-like compounds. This, broadly speaking, is true if the irreversibly inhibitory organic phosphates are left out of account. There is one well-recognized exception ; that is the true cholinesterase inhibitor Nu 1250 (Hawkins and Mendel, 1949), which has the following structure:



In recent years Nachmansohn and Whittaker and their co-workers have reached certain conclusions regarding the chemical nature of the active centres of the two cholinesterases ; the main difference between them seems to lie in the possession by the true cholinesterase of one or more anionic groups which are absent from the pseudo cholinesterase. Bergmann, Wilson, and Nachmansohn (1950) suggest, on the basis of experiments comparing the inhibition of mono- and bis-quaternary salts, that true cholinesterase probably possesses two anionic groups capable of binding quaternary nitrogen groups separated by a distance of 12–14 Å—that is, structures similar to the basic structure of the most potent neuromuscular blocking drugs (cf. also Paton and Zaimis, 1953). That the neuromuscular blocking drugs appear to be specific inhibitors of true cholinesterase is additional evidence for the view put forward by these workers.

An idea, which seems to be implicit in their conclusions that the active centre of true cholinesterase must resemble in structure the acetylcholine receptor at the neuromuscular junction, was first suggested in rather more general terms by Roepke in 1936 (cf. Wescoe and Riker, 1951).

In an analogous manner, it could be argued that the correlation of antimuscarinic activity with specificity for the pseudo cholinesterase reflects a structural resemblance between the acetylcholine receptors in the parasympathetic nervous system and the active centre of the pseudo cholinesterase. It is perhaps of interest, though the connexion is by no means clear, that Ord and Thompson (1950) have found that pseudo cholinesterase is present in excess of acetylcholinesterase in those tissues of the rat in which acetylcholine exerts a muscarinic action ; further, Koelle, Koelle, and Friedenwald (1950) and Burn, Kordik, and Mole (1952) have suggested that pseudo cholinesterase is of physiological significance in the intestine of the rat.

Summary

1. The inhibition of the cholinesterases of rat brain and intestinal mucosa by a series of 46 pharmacologically active compounds has been studied.

2. The ratio of the 50% inhibition concentrations for the two enzymes tends to show some correlation with the predominant pharmacological action of the compound ; thus, for most neuromuscular blocking agents the ratio [I]50 brain/[I]50 intestinal mucosa has a value less than 0.2 ; for antimuscarinic agents the ratio lies between 5 and 60 ; while for drugs effective in Parkinsonism the ratio is greater than 40.

3. Two neuromuscular blocking drugs possess ratios greater than unity. One, gallamine triethiodide, also exhibits antimuscarinic activity ; the other, IS302, has a chemical structure intermediate between those of neuromuscular blocking and antimuscarinic drugs.

4. Most antihistaminic drugs show no tendency to inhibit either enzyme specifically ; three, which exhibit marked antimuscarinic action, have higher ratios than the average.

5. Hyoscine does not show any correlation between pharmacological action and specificity of cholinesterase inhibition.

6. The significance of these findings is discussed.

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THE ACTION OF ESERINE SULPHATE ON THE SPINAL CORD OF THE CAT

BY

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Feldberg (1951), reviewing the pharmacology of central synaptic transmission, classified the evidence in favour of the hypothesis that acetylcholine acts as a central transmitter under three headings: 1, direct effects of acetylcholine on the central nervous system; 2, the acetylcholine content of nervous tissue; 3, the action of anticholinesterases on the activity of the central nervous system.

Much of the evidence for the last has been obtained from the study of the action of eserine on spinal reflex activity in mammals, using the classical methods of reflex elicitation and recording. A review of this evidence shows general agreement that eserine has a stimulant action on spinal reflex activity; but there are differences concerning its action on individual reflexes, and on the influence of the previous administration of atropine. Some of this conflict is evidently due to differences in the preparations used and the mode of administration of the drug, but it has also been noted that the action of eserine is irregular and unpredictable (Calma and Wright, 1944).

Schweitzer and Wright (1937) found that the intravenous injection of eserine in doses of 0.03–0.2 mg./kg. into cats anaesthetized with chloralose markedly increased the knee jerk and general reflex excitability. Calma and Wright (1947) reported similar changes in the knee jerk after the intrathecal administration of eserine, but found variable changes in the flexor reflex. Merlis and Lawson (1939) stated that both intravenous and intrathecal administration of eserine depressed the knee jerk and augmented the flexor reflex; similar results were reported by Bülbring and Burn (1941) using intra-aortic injection in the dog.

This paper reports investigations into the action of eserine on the spinal cord of the cat, using methods developed in previous studies of the action of drugs on the central nervous system (Bernhard and Taverner, 1951; Bernhard, Taverner, and Widén, 1951; Taverner, 1952, 1953). Monosynaptic and polysynaptic reflex discharges and cord dorsum potentials were evoked by electrical stimulation of appropriate afferent fibres in motor and sensory nerves in unanaesthetized preparations. The only previous report on the action of eserine, using comparable methods, is that of Wikler (1945), who found that the intravenous injection of eserine into spinal cat preparations increased the amplitude of monosynaptic flexor and extensor reflex responses and had no effect on polysynaptic flexor reflex responses.

MATERIALS AND METHODS

Cats were used in all experiments. Decerebration or spinal section was carried out under ether anaesthesia, but the effects of the latter had disappeared when the experiments were performed two or three hours later. All dissection and manipulation of nervous structures were performed under warm paraffin oil. The standard electro-physiological technique employed has been described (Bernhard and Taverner, 1951; Taverner, 1952). The rate of stimulation was once every four seconds. All the drugs employed were injected slowly through an indwelling polythene tube which was passed up the superior mesenteric artery so that its tip lay in the aorta above the origins of the lumbar vessels. The drugs used in various experiments were freshly prepared eserine sulphate (0.1%), strychnine hydrochloride (0.01%) and pentobarbitone sodium (Nembutal) (0.5%).

RESULTS

Electrical stimulation of low threshold afferent fibres (Group 1) in the gastrocnemius nerve is followed, after an interval of 2.5–3.0 msec., by a synchronous reflex discharge in the S1 ventral root (Fig. 1A). It has been shown by Lorente de Nó (1935), Renshaw (1940), and Lloyd (1943) that this discharge represents activity in two neuronal (monosynaptic) reflex arcs and returns to the muscle from which the afferent fibres arise. Myotatic reflexes—tendon jerks—are probably of this type. If the stimulus is applied to a cutaneous

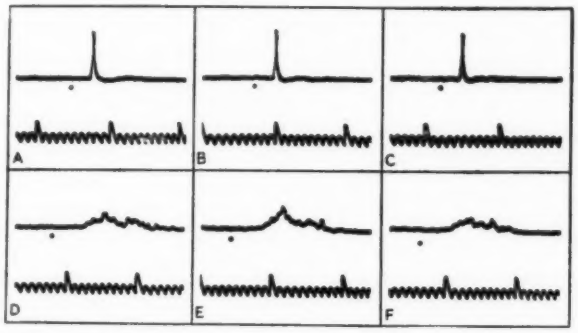

FIG. 1.—Effect of eserine on the monosynaptic extensor (upper series) and polysynaptic flexor (lower series) reflex responses. A, D, controls. B, E, 1 min., C, F, 20 min., after intra-aortic injection of 0.1 mg. eserine sulphate per kg. Dots indicate moment of stimulation. Time in msec.

nerve, such as the sural, an asynchronous, irregular discharge appears in the S1 ventral root after 4–5 msec. and persists for 10 msec. or more (Fig. 1D). Lloyd (1943) has shown that this discharge represents activity in more complex reflex arcs with several interpolated internuncial neurones (polysynaptic reflexes); flexor and crossed extension reflexes are believed to depend on such polysynaptic chains.

Effect of Intravenous Eserine on Spinal Reflexes in Unanaesthetized Preparations

In a series of preliminary experiments eserine sulphate, 0.1–0.2 mg./kg. body weight, was administered by slow intravenous injection to decerebrate and to high and low spinal preparations. The polysynaptic reflex response increased in amplitude to 130–150% of the pre-injection value in all types of preparation. The latency of the effect varied from 30 to more than 150 seconds. In decerebrate preparations there was an increase in the amplitude of the monosynaptic extensor reflex response in all the experiments save one. In preparations with spinal cord section at various levels the results were irregular and unpredictable, but the monosynaptic extensor reflex response was rarely augmented by eserine; it was mostly either unaffected or reduced in amplitude. Analysis of these results suggested that the variability was due to the hypotensive effect of intravenous eserine. In the decerebrate preparations this was of little significance because of the relatively high initial blood pressure values. In preparations with spinal section the initial blood pressure was usually between 50–60 mm. Hg, and a fall to 40 mm. Hg or less was frequently observed. It is known that a blood pressure level of 40 mm. Hg is critical for the functioning of the spinal reflexes (Taverner, 1953).

Effect of Intra-aortic Eserine on the Spinal Reflexes in Unanaesthetized Preparations

Consistent results were obtained in a series of experiments where the eserine was injected slowly through a polythene tube inserted into the aorta via the superior mesenteric artery so that its tip lay above the site of origin of the lumbar arteries. This tube was filled with normal saline between injections and was controlled with a three-way tap from a Greenfield lumbar puncture needle.

Fig. 1 shows the effect of 0.1 mg./kg. eserine injected into the aorta of a decerebrate cat with the spinal cord sectioned in the upper cervical region. The reflexes were sampled alternately at 2 sec. intervals. Fig. 1A shows the monosynaptic extensor reflex response evoked by electrical stimulation of the gastrocnemius nerve, and Fig. 1D the polysynaptic reflex response elicited from the sural nerve, before the administration of eserine. Figs. 1B and 1C show that the monosynaptic reflex response is unchanged 1 min. and 20 min., respectively, after the eserine. The polysynaptic reflex response is increased 1 min. later (Fig. 1E), but has returned to its pre-injection level 20 min. later (Fig. 1F). Doses of eserine of less than 0.1 mg./kg. body weight had no significant effect on either reflex under these experimental conditions.

Larger doses of eserine produced more striking results. Figs. 2A and D show the monosynaptic extensor and polysynaptic reflex responses, respectively, before the administration of 0.2 mg./kg. body weight of eserine by intra-aortic injection in a decerebrate preparation with high spinal section. Figs. 2B and E show the two reflexes 1.5 min. after the injection, and Figs. 2C and F 30 min. after the injection. Both reflexes nearly doubled in size shortly after the injection.

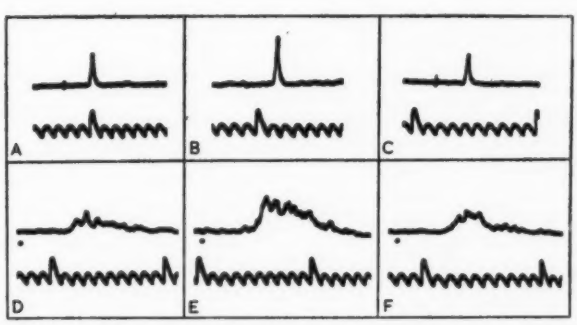

FIG. 2.—Effect of eserine on the monosynaptic extensor (upper series) and polysynaptic flexor (lower series) reflex responses. A, D, controls. B, E, 1.5 min., C, F, 30 min., after intra-aortic injection of 0.2 mg. eserine per kg. Time in msec.

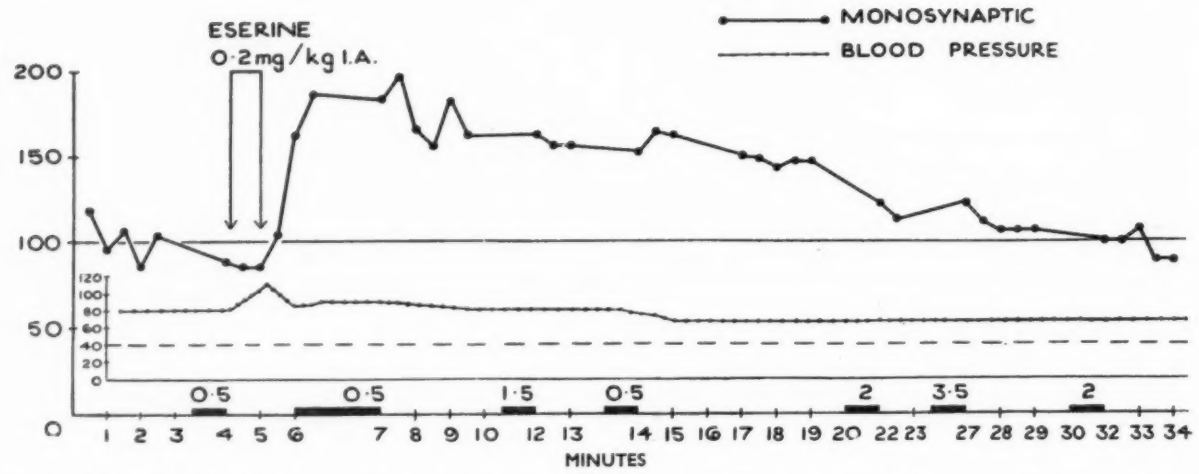

FIG. 3.—Effect of 0.2 mg./kg. eserine sulphate on the monosynaptic extensor reflex response and blood pressure. (Same experiment as Fig. 2.) Ordinates: Reflex response as percentage of pre-injection amplitude (main graph). Blood pressure in mm. of mercury (lower inset graph). Abscissae: Time in min. Bars on base line represent suspension of recording.

The time course of these changes is demonstrated in Figs. 3 and 4. In Fig. 3 the amplitude of the monosynaptic extensor reflex response is expressed as a percentage of the mean pre-injection level. Sampling of the reflex continued throughout the injection of eserine. Thirty seconds after the end of the injection the amplitude of the monosynaptic reflex response increased rapidly to 185% of the pre-injection level. The increase persisted for 2 min. and then, after a fairly rapid fall in amplitude, a level of 160% was maintained for a further 10 min. The monosynaptic reflex then declined slowly in amplitude and returned to the pre-injection value 30 min. after the injection. The blood pressure was recorded throughout and rose during the injection from a previously steady level of 80 mm. Hg to 110 mm. Hg. During the next minute it fell again to 90 mm. Hg and 4 min. later had reached the original level of 80 mm. Hg. Nine minutes after the injection the blood pressure fell to 70 mm. Hg and remained at this level until the end of the experiment.

Fig. 4 shows the changes in the polysynaptic reflex response in the same experiment. The results are expressed in the same way as in Fig. 3. The augmentation of the polysynaptic reflex response after the intra-aortic injection of 0.2 mg./kg. eserine is almost identical with that of the monosynaptic reflex response. The initial increase to 190% of the pre-injection level began 30 sec. after the end of the injection.

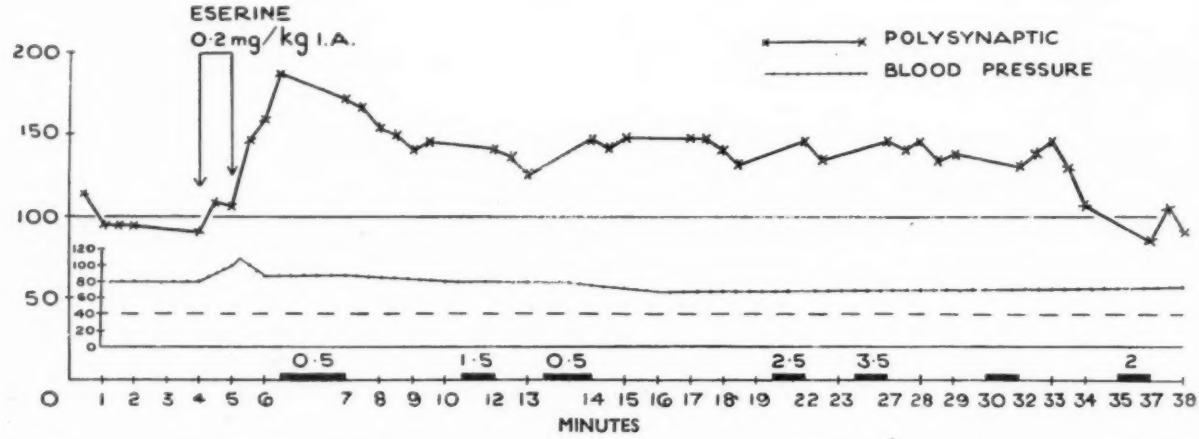

FIG. 4.—Effect of eserine on the polysynaptic flexor reflex response. (Same experiment as Figs. 2 and 3.) Ordinates and abscissae as in Fig. 3.

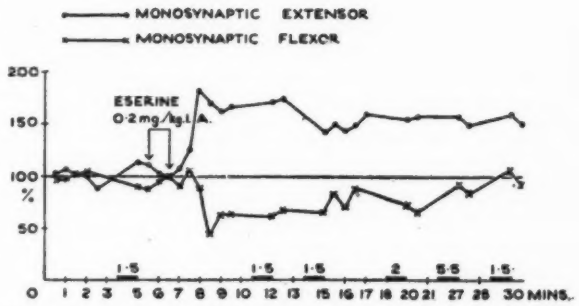

FIG. 5.—Effect of 0.2 mg./kg. eserine on the monosynaptic extensor (filled circles) and monosynaptic flexor reflex responses. Ordinate: reflex response as percentage of pre-injection amplitude. Abscissa: time in min.

Similar increases in both the monosynaptic extensor and polysynaptic reflex responses occurred in all experiments with decerebrate and with high or low spinal preparations when the eserine was given by intra-aortic injection. The arterial blood pressure always rose during the intra-aortic injection of eserine, in contrast to the fall in blood pressure produced by intravenous injection.

Effect of Intra-aortic Eserine on the Monosynaptic Flexor Reflex in Unanaesthetized Preparations

Monosynaptic reflex responses can also be elicited by electrical stimulation of low threshold fibres in the nerves supplying flexor muscles of the limbs. Such monosynaptic flexor reflexes may be affected by drugs in a different manner from monosynaptic extensor reflexes (cf. Bernhard and Taverner, 1951).

Fig. 5 shows the effects of the intra-aortic injection of eserine on the monosynaptic extensor reflex evoked by stimulation of muscle afferent fibres in the nerve to one head of the gastrocnemius muscle and on the monosynaptic flexor reflex elicited from the nerve to the hamstring muscles in a decerebrate preparation with high spinal section. The injection of 0.2 mg./kg. eserine increases the amplitude of the monosynaptic extensor reflex response in the manner already described (Figs. 2 and 3). Coincident with the rise in the extensor response there is a rapid fall in the amplitude of the monosynaptic flexor response to less than 50% of its pre-injection value. This reduction is maintained for some minutes and the flexor reflex then slowly regains its normal size, which it reaches after about 25 min., when the extensor response is still 150% of the pre-injection level.

Effect of Intra-aortic Eserine in De-afferented Preparations

The injection of an anticholinesterase such as eserine leads to widespread muscular twitching. It is possible that afferent impulses during this period might lead to changes in the excitability of the spinal cord and thus account for the alterations in the reflex responses described above.

Control experiments were therefore performed in which the spinal cord was cut at the junction of the T12–L1 segments and all the dorsal roots on both sides from L2 downwards were sectioned intradurally. In addition the L7 and S1 ventral roots were severed intradurally. Electrical stimulation of the S1 dorsal root produces a complex reflex discharge in the S1 ventral root composed of monosynaptic and polysynaptic elements. The intra-aortic injection of eserine into such preparations increased the amplitude of the polysynaptic reflex response to approximately twice the pre-injection level. The monosynaptic reflex response was depressed to a varying degree, but both reflexes had returned to their pre-injection values 20 min. later. The monosynaptic reflex response probably falls because afferents of the monosynaptic flexor reflex are also travelling in the S1 dorsal root (cf. Fig. 5). This response of the spinal cord isolated from afferent impulses suggests that the action of eserine is central.

Effect of Intra-aortic Eserine on the Cord Dorsum Potentials

It has been known for many years that electrical stimulation of cutaneous afferent fibres produces potential changes in the spinal cord (Gotch and Horsley, 1891; Gasser and Graham, 1933; Barron and Matthews, 1938; Lloyd and McIntyre, 1944; and Eccles, 1946). When the sural nerve is stimulated records obtained from a monopolar electrode resting on the dorsal surface of the S1 segment of the spinal cord consist of an initial negative wave followed by a varying degree of positivity. Bernhard (1953) has suggested that the first wave of negativity represents activity in monosynaptically

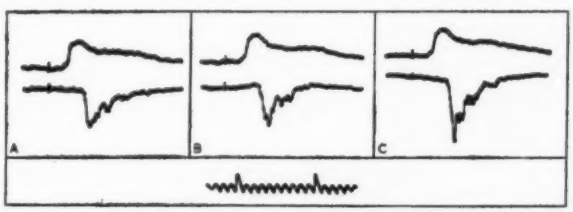

FIG. 6.—Effect of eserine on the cord dorsum potentials (upper series) and polysynaptic flexor reflex response (lower series) elicited by electrical stimulation of the sural nerve. A, control. B, 1 min., C, 18 min., after 0.18 mg. eserine sulphate per kg. by intra-aortic injection. Polysynaptic response recorded negativity downwards. Time in msec.

activated cell bodies in the dorsal grey matter concerned in the upward transmission of afferent impulses. The inconstant positivity, which can be greatly accentuated by the intravenous injection of strychnine, originates from propriospinal interneurones extending over several segments of the spinal cord, which are not essential for reflex transmission.

Fig. 6A shows the cord dorsum potentials (upper beam) evoked by electrical stimulation of the sural nerve in a decerebrate preparation with high spinal section. The lower beam shows the polysynaptic reflex response simultaneously recorded from the S1 ventral root. Fig. 6B records the two responses 1 min. after the intra-aortic injection of 0.18 mg./kg. eserine. The cord dorsum potentials are unchanged, but the polysynaptic reflex response is beginning to increase in size. 18 min. after the injection (Fig. 6C) the cord dorsum potentials are unchanged, but the polysynaptic reflex response is now considerably augmented. In further experiments it was found that eserine did not modify the cord dorsum potentials even after the positive component had been greatly increased by the administration of strychnine.

Influence of the Administration of Pentobarbitone and Atropine on the Action of Eserine

Fig. 7A shows the control monosynaptic extensor reflex response evoked by electrical stimulation of the nerve to the gastrocnemius muscle in a decerebrate preparation. Fig. 7B records the reduction in size of the reflex 45 min. after the intravenous injection of 5.5 mg./kg. of pentobarbitone sodium. Between records 7B and 7C, 0.2 mg./kg. eserine was injected intravenously and 3 min. later (Fig. 7C) the reflex response had returned to its original size. This increase was short-lived, and 15 min. after the eserine injection the monosynaptic reflex was again in its depressed state. The time course of these changes is shown in Fig. 8. The monosynaptic reflex response is plotted in terms of the pentobarbitone-depressed response as 100%. The injection of 0.2 mg./kg. eserine doubles the size of the monosynaptic reflex response, but the effect is short-lived and has almost disappeared 13 min. later.

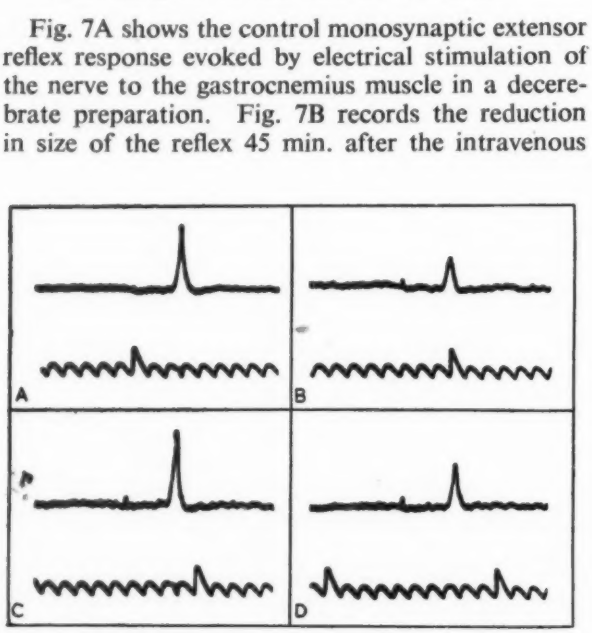

FIG. 7.—Effect of eserine on the monosynaptic extensor reflex response depressed by pentobarbitone sodium. A, control. B, 45 min. after 5.5 mg. pentobarbitone per kg. by intra-aortic injection. C, 3 min. after 0.2 mg. eserine sulphate per kg. by intra-aortic injection. D, 15 min. after eserine. Time in msec.

In some experiments atropine sulphate was injected intravenously, or into the aorta, before the administration of eserine. 0.75 mg./kg. atropine sulphate injected into the aorta abolished the effect of a subsequent dose of 0.2 mg./kg. eserine

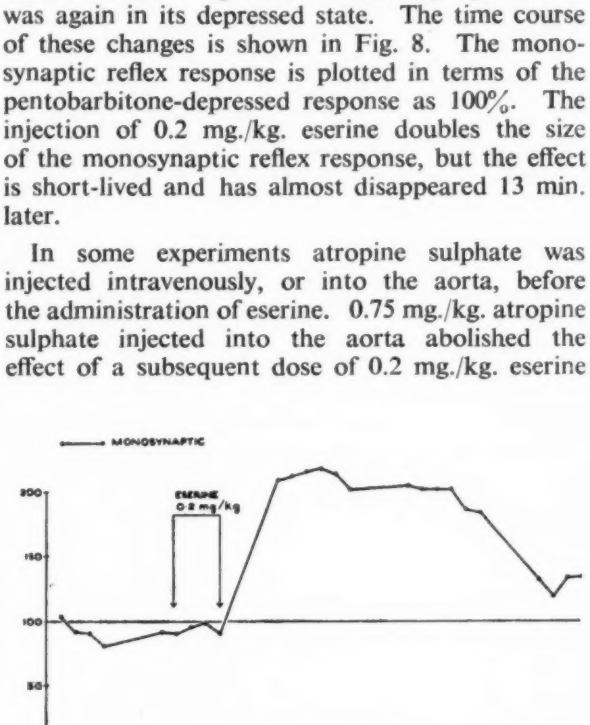

FIG. 8.—Effect of intra-aortic eserine on the monosynaptic reflex depressed by the administration of 5.5 mg. pentobarbitone per kg. 45 min. previously. Ordinate: reflex response as percentage of pre-injection amplitude. Abscissa: time in min.

on the monosynaptic and polysynaptic reflex responses in a high-spinal preparation. Smaller doses of atropine diminished the central action of eserine.

DISCUSSION

The initial experiments with intravenously administered eserine gave unpredictable results, confirming the findings of Calma and Wright (1944). The only exception was in decerebrate preparations in which both the monosynaptic extensor and polysynaptic reflex responses were regularly augmented by intravenous eserine. These results were probably due to the hypotensive action of intravenous eserine, which in the spinal preparations frequently lowers the blood pressure to 40 mm. Hg—the critical value

for satisfactory reflex responses. The consistent results obtained after the intra-aortic injection of eserine, which raised the blood pressure, enabled a more accurate analysis of the action of eserine on the central nervous system to be made. The method of approach through the superior mesenteric artery proved more satisfactory than the insertion through the femoral artery employed by Skoglund (1953) which occasionally interfered with the blood supply to the site of stimulation in the leg.

The results obtained confirm and amplify the findings of Schweitzer and Wright (1937). Eserine doubles the size of both the monosynaptic extensor and polysynaptic reflex responses in all types of preparation used, and this effect is not abolished by the previous administration of a barbiturate in doses which markedly depress the spinal reflexes. The differences between the present results and those of Merlis and Lawson (1939) and Bülbring and Burn (1941) are probably attributable to the different preparations and mode of administration of the drug which were employed. The results of Wikler (1945), who used comparable electrophysiological methods, agree with the present findings only in respect of the increase of the monosynaptic extensor reflex response after eserine administration. Wikler reported no effect on the polysynaptic flexor reflex response, and an increase in the monosynaptic flexor response, in contrast to the decrease in the amplitude of the latter reflex which was found in this investigation. The discrepancy can hardly be attributed to the different method of drug administration used.

Although the stimulant effect of eserine sulphate on spinal cord activity appears to be established, there remains the possibility that its action is a direct one and does not depend on its anticholinesterase properties. It is probably significant that other anticholinesterases, such as tabun (Skoglund, 1953) and di*iso*propyl fluorophosphonate (Chennels, Floyd, and Wright, 1951), have been shown to have a similar effect to eserine on the spinal cord. Further, Schweitzer, Stedman, and Wright (1939) showed that the conversion of eserine to eseroline, by the removal of the urethane grouping said to be responsible for the anticholinesterase activity, also eliminated the action of eserine on the spinal cord. Again the abolition of the central effects of eserine by the prior administration of atropine also favours the view that this central action is a manifestation of the anticholinesterase activity of eserine.

If this last view be accepted, then it is evident that not all central synaptic activity can be related to the presence of acetylcholine. Most forms of spinal reflex activity are augmented by eserine, but the cord dorsum potentials are unaffected. This is to be expected for the N1 potential—which derives from monosynaptically activated cells in the dorsal grey matter concerned in afferent transmission—because it is known that posterior roots contain no acetylcholine or choline acetylase (Feldberg, 1951). The primary sensory fibres are unlikely, therefore, to possess cholinergic endings. The P component of the cord dorsum potentials might be expected to respond to eserine, because the P potential is believed to represent interneuronal activity, and Feldberg, Gray, and Perry (1953) have suggested that intra-arterially administered acetylcholine acts upon the interneurones. However, these authors used different methods and did not study the effect of acetylcholine on the cord dorsum potentials. The potential cannot be regarded as a quantitative indicator of interneuronal activity and very probably derives from only part of the spinal interneurones. The present results may imply that these neurones are not cholinergically activated; but Skoglund (1953) showed that the powerful anticholinesterase tabun abolished the P component, and this may have been due to the activation of excessive amounts of acetylcholine, giving a depressant effect. Investigation of the central effects of other anticholinesterases may help to explain these discrepancies.

Summary

1. The action of eserine sulphate on the activity of the spinal cord in the cat has been investigated in unanaesthetized, decerebrate, and spinal preparations.

2. Intravenous administration was found to be unsatisfactory because of the effect on the blood pressure, but intra-aortic injection gave consistent results.

3. Intra-aortic eserine sulphate increases the amplitude of the monosynaptic extensor and polysynaptic flexor reflex responses and decreases the amplitude of the monosynaptic flexor reflex response. It has no effect on the cord dorsum potentials evoked by electrical stimulation of cutaneous afferent nerve fibres.

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PHARMACOLOGY OF THE PORPHYRINS AND PORPHOBILINOGEN

BY

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This paper attempts to define the relationship between the abnormal substances excreted in acute porphyria and the clinical manifestations of this disorder of porphyrin metabolism. During an attack of acute porphyria, patients usually excrete large quantities of porphobilinogen either alone or with certain porphyrins. The excretion of the porphyrins and porphobilinogen is usually in direct proportion to the severity of the symptoms, suggesting a causal relation, although Waldenström (1939) has reported an authenticated case in which the patient did not pass uroporphyrin or porphobilinogen in the urine or bile during the attack, but did so on other occasions. Several authors have claimed that porphyrins may influence the intestine or uterus (Supniewski, 1927; Gunther, 1922; Reitlinger and Klee, 1928; Vannotti, 1937; and Simici, 1938). Critical appraisal of these reports has led us to repeat this work using porphyrins of the kind known to be excreted in porphyria, which were obtained by improved methods of purification. The isolation of porphobilinogen in crystalline form (Westall, 1952) has for the first time allowed pharmacological testing of the pure substance, although Waldenström and Wendt (1939) and Prunty (1945) had injected partially purified porphobilinogen into rabbits.

METHODS

Animal Experiments.—Observations were made on the blood pressure (recorded with a cannula in the carotid artery) and on the respiration of 13 cats and 3 rabbits (anaesthetized with chloralose (80 mg./kg.) after induction with ether) and of 1 pithed cat, 1 pithed and eviscerate cat, and 1 decerebrate cat. Injections were made into the right femoral vein or the splenic vein.

Stimulation of the distal end of the vagus, separated and cut in the neck, was with supramaximal 0.5 msec. shocks at 10 c./s.

Isolated Organs.—Experiments were also made on isolated strips of guinea-pig ileum, non-pregnant rabbit uterus, rabbit jejunum and ileum, or cat ileum, set up in Tyrode's solution at 34° C. Contractions were recorded on smoked paper by a frontal writing lever. Experiments with light irradiation were done with an electric bulb of 300 w. at 25 cm. from the tissue in the organ bath.

Drugs.—The porphyrins, with the exception of haematoporphyrin, had been isolated from biological material as the methyl esters. Before use the esters were hydrolysed with 7 N HCl for 36 hours, at room temperature, the excess of HCl being then removed in a vacuum desiccator over KOH. Haematoporphyrin was prepared and used as the dihydrochloride. Pure crystalline porphobilinogen (Westall, 1952) was used; the porphobilin was obtained by Mr. R. G. Westall as a by-product in the preparation of porphobilinogen.

Porphobilinogen was dissolved in a minimum volume of 0.1 N $NH_4.OH$ and then made up to the required volume with 0.9% saline. For the porphyrins and porphobilin M/7 sodium bicarbonate was used as the solvent.

RESULTS

Anaesthetized Cats and Rabbits.—Recordings were made of the direct effect of porphobilinogen—and, in one experiment, of uroporphyrin I—on the blood pressure, respiration and vascular responses of the treated animals to acetylcholine, histamine, nicotine, adrenaline, noradrenaline, and vagal stimulation. The amount of porphobilinogen injected (up to 100 μg./kg.) was limited by the amount available; but from the known rate of excretion in patients (40–160 mg./day), and from the fact that tests for plasma porphobilinogen sensitive to 1 μg./ml. may be negative even at the height of an attack, it is likely that the blood levels obtained in our experiments were comparable to or even greater than those obtaining in acute porphyria. The only effect observed was an apparent potentiation by porphobilinogen of the response to adrenaline and noradrenaline in a few of the early

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experiments (Fig. 1). This apparent potentiation of adrenaline and noradrenaline could not, however, be repeated and its interpretation is complicated by the fact that considerable spontaneous fluctuations in sensitivity to these drugs may occur.

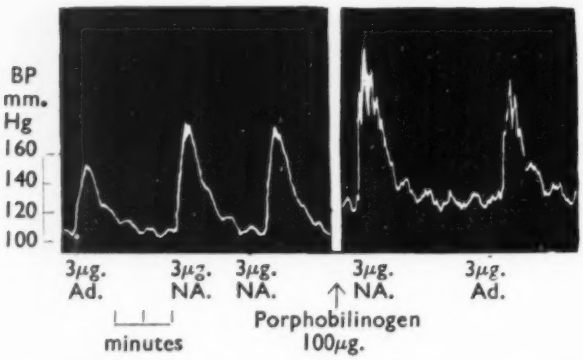

FIG. 1.—Cat.; chloralose; blood pressure recording; intravenous injections. Responses to 3 μg. adrenaline and noradrenaline before and after 100 μg. porphobilinogen.

Isolated Organs.—After obtaining records of spontaneous activity and tone, and of consistent responses to acetylcholine, histamine, adrenaline, and 5-hydroxytryptamine, the effect of adding porphyrins, porphobilinogen, and porphobilin to the preparation was investigated. With uroporphyrin I the effect of light irradiation was also determined. A summary of these results is given in Table I.

The only significant responses were those to haematoporphyrin (1/8,000) and to porphobilin. The former produced a distinct waning contraction of guinea-pig ileum, followed by inactivity of the intestine and a refractoriness—which became complete—to histamine and acetylcholine. Rabbit intestine was unaffected. Porphobilin produced a histamine-like contraction, sensitive to mepyramine, but less so to atropine (Fig. 2); it was considered likely that the effect was due to contamination (c. 0.4 mg./g.) with histamine itself.

Test of Porphobilinogen on Unanaesthetized Rabbit.—10 mg. porphobilinogen was injected intravenously into a rabbit (2.2 kg.) with an external biliary fistula. The animal showed no abnormal symptoms in the 3 days following the injection. There was a slight rise in the level of bile protoporphyrin during this period and a trace of uroporphyrin was noted in the urine several hours after the injection. No porphobilinogen was found in the urine.

Test of Whole Urine from Patients with Acute Porphyria.—As a final test, to cover the possibility that in porphyria some unidentified pharmacolo-

TABLE I
TESTS OF PORPHOBILINOGEN AND PORPHYRINS ON ISOLATED TISSUES

		Porphobilinogen	Uroporphyrin I	Uroporphyrin III	Coproporphyrin I	Coproporphyrin III	Haematoporphyrin	Porphobilin
Rabbit uterus	Drug concn.	1/40,000	1/10,000	1/40,000	1/40,000	1/40,000		
	Effect on spontaneous activity and tone	0	0	0	0	0		
	Effect on response to adrenaline	0	0	0	0	0		
Rabbit ileum or jejunum	Drug concn.	1/40,000	1/10,000				1/10,000	
	Effect on spontaneous activity and tone	0	0				0	
	Effect of combining with light irradiation		0					
	Effect on response to histamine, acetylcholine, adrenaline						0	
Guinea-pig ileum	Drug concn.	1/20,000	1/40–10,000	1/10,000	1/20,000	1/20,000	1/8,000	1/20,000
	Effect on spontaneous activity and tone	0	Slight + twice only	0	+ twice only	0	–	+ (histamine contaminant ?)
	Effect on response to acetylcholine	0					–	
	Effect on response to 5-OH tryptamine	0		0	0	0		
	Effect on response to histamine		+ (50%) once only				–	

0 = No effect: + = increase; – = decrease.

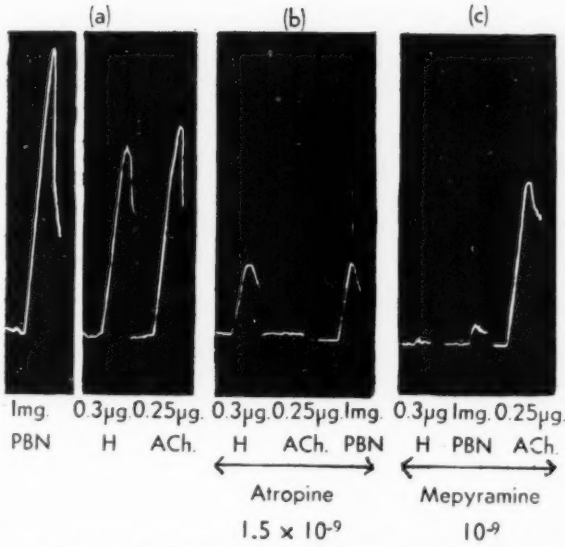

FIG. 2.—Isolated guinea-pig ileum. Response to 1 mg. porphobilin, 0.3 μg. histamine and 0.25 μg. acetylcholine (a) normally, (b) in presence of 1.5×10^{-9} atropine, (c) in presence of 10^{-9} mepyramine.

gically active substance is excreted, a sterile specimen of urine from a patient suffering a moderately severe attack of acute porphyria (with hypertension and abdominal pain) was infused into an anaesthetized cat at a rate of approximately 4 ml./min. for 15 min. This by itself produced no effect on blood pressure or respiration; and its effect on the responses to adrenaline, noradrenaline, histamine, acetylcholine, and nicotine was indistinguishable from that of a specimen of normal urine.

DISCUSSION

Despite a great deal of research on acute porphyria the mechanism of the production of symptoms remains obscure. Recent work has tended to minimize the possible direct influence of the porphyrins (Waldenström, 1939) and to emphasize the importance of the pathological features of patchy myelin change observed in the peripheral and autonomic nerves (Denny-Brown and Sciarra, 1945). These authors considered that the changes might be caused by an intermittent ischaemia, probably due to a circulating vasoconstrictor substance. Following this, Wehrmacher (1952) reported clinical improvement in acute porphyria with the use of ganglion-blocking agents. A search for some such vasoconstrictor substance, which might be present only in active cases of acute porphyria, would be a reasonable approach to the problem. The pharmacological testing of the known and already purified excretion products was clearly necessary. It would be unlikely that uroporphyrin and coproporphyrin could fulfil this role, since these are excreted in increased amounts in both congenital porphyria (as the series I isomers), and in porphyria cutanea tarda (as the series I and III isomers) where skin photosensitivity may be the only symptom. Porphobilinogen, however, is always excreted in the urine in attacks of acute porphyria and in those phases of porphyria cutanea tarda where acute symptoms are superimposed on the cutaneous syndrome. For this reason porphobilinogen or some closely related substance has been strongly suspected of being the *materia peccans* of acute porphyria (Lowry *et al.*, 1950).

Our experiments have failed to show that either the porphyrins or porphobilinogen have any significant pharmacological action. The initial animal experiments, in which porphobilinogen appeared to potentiate the blood pressure responses of the cat to adrenaline and noradrenaline, could not be repeated. These interesting results cannot be explained, although it is just possible that there may be an individual tissue and animal sensitivity to such drugs. Their inactivity in our hands is at variance with the results of some previous investigators. The difficulty of isolating porphyrins from biological materials, such as urine, in a state of purity that will guarantee freedom from possible histamine contamination, is very great. Our experience suggests that the contradictory results obtained by previous workers may possibly have been due to histamine contamination.

The results of intravenous injection of porphobilinogen into a rabbit confirm Prunty's (1945) findings, but contradict those of Waldenström and Wendt (1939), who found porphobilinogen in the urine of a rabbit into which they had previously injected the partially purified substance (amount used unknown).

Further work has tended to substantiate the absence of pharmacological activity of the porphyrins and porphobilinogen. An "experimental porphyria" or disturbance of porphyrin metabolism has been produced in rabbits by the non-hypnotic substance allyl *iso*propyl acetamide (Goldberg, 1953); very large quantities of uroporphyrin and porphobilinogen were excreted—in one animal for as long as three weeks—without obvious pharmacological effect. Apart from constipation, there is no evidence that the state of these rabbits compared with the clinical state of human acute porphyria, although the animals excreted proportionately greater quantities of the substances. Falk, Dresel, and Rimington (1953) have shown that porphobilinogen is a porphyrin precursor in a tissue system. This emphasizes that in porphobilinogen

we are probably dealing with a physiological substance. While our experiments therefore do not rule out the possibility that an unidentified vasoconstrictor substance may be produced in acute porphyria, or that the known excretion products may exert a pressor action by a mechanism at present unknown, they render these suggestions unlikely.

Summary

1. The porphobilinogen and the porphyrins usually excreted in acute porphyria, as well as haematoporphyrin and porphobilin, have been tested pharmacologically.

2. Apart from slight and variable action of uroporphyrin I, coproporphyrin I, and porphobilin, these substances show no pharmacological action.

3. The significance of the results in relation to the symptoms of acute porphyria is discussed.

We wish to thank Mr. R. G. Westall for generous supplies of porphobilinogen and Professor C. Rimington for specimens of porphyrins, and constant help through these experiments.

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FACTORS INFLUENCING THE EFFECT OF MORPHINE SULPHATE ON THE ASCORBIC ACID CONTENT OF RATS' ADRENAL GLANDS

BY

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In 1923 Lewis showed that morphine was 400–500 times more toxic to adrenalectomized than to normal rats. This suggests that the drug stimulates the adrenal cortex; it has since been shown (Nasmyth, 1948) that the subcutaneous injection of morphine causes a depletion of the ascorbic acid content of rats' adrenals. Morphine also affects the secretion of the adrenal medulla; Elliott, in 1912, demonstrated that the pressor amine content of the cat's adrenal was much reduced by morphine. More recently, Emmelin and Strömblad (1951) showed a similar reduction in the adrenaline and noradrenaline content of the cat's adrenal. In 1952 Evans, Nasmyth, and Stewart showed that after intravenous injection of morphine the pressor activity of blood samples from the adrenal vein of the cat was increased tenfold for more than 10 minutes. By direct measurement, Vogt (1944) has shown that the injection of adrenaline causes an increased secretion of cortical hormone in the dog, and various authors have described the depletion of the ascorbic acid content of the rat's adrenal glands when adrenaline is injected subcutaneously (Long and Fry, 1945; Nasmyth, 1950; and Jarrett, 1951).

Morphine can also liberate histamine (Feldberg and Paton, 1950 and 1951; and Nasmyth and Stewart, 1950) and a relationship between histamine and the activity of the adrenal cortex was indicated by Gottesman and Gottesman (1928), who showed adrenalectomized rats to be 20 times more sensitive to histamine than normal animals. Binet (1941) showed that histamine causes a release of adrenaline from the adrenal medulla, thus confirming the suggestion of Dale (1920) and the work of Kellaway and Cowell (1922). Histamine causes a depletion of ascorbic acid in the rat adrenal cortex (Sayers and Sayers, 1947; Nasmyth, 1951; and Halpern and Benos, 1952), and this effect is independent of its ability to release adrenaline from the adrenal medulla (Nasmyth, 1951).

Since morphine can liberate both adrenaline and histamine, it was of interest to determine how much these two substances contribute to the effect of morphine on the adrenal cortex.

METHODS

Administration of Drugs.—Litter mate, male rats of either the Hooded or Wistar strains were used. The Hooded strain was obtained from Mr. Locke's colony at Crooke's Laboratories and the Wistar strain from Dr. Mandl's colony at Birmingham University, and from the colony at Glaxo Laboratories. These strains were distributed as evenly as possible between the various groups of animals used in the following experiments. The rats were on a stock diet.

In each experiment 2 mg./100 g. body weight of morphine sulphate was injected subcutaneously in the test animals, whereas the controls, taken from the same litter, were injected with an equal volume of normal saline. The test animals were killed by a blow on the back of the head 1½, 3, and 5 hr. after the injection; the controls were killed between 1 and 3 hr. after the saline dose, as Jarrett (1951) has shown the effect of saline on the rat's adrenal ascorbic acid to be maximal between these times. Immediately after each rat was killed, the abdomen was shaved and a piece of skin weighing between 0.5 and 1 g. was taken for determination of its histamine content.

Extraction of Glands.—The adrenal glands were removed, dissected free of superfluous tissue, and weighed to 0.0002 g. This procedure occupied approximately the same time in each experiment, so that any loss in weight due to drying could be assumed to be approximately constant. Each gland was then ground in a test-tube containing 10 ml. of 6% trichloracetic acid and a little acid-washed sand, with the aid of a glass rod having an end shaped to fit the bottom of the tube. This was sufficient to extract all the ascorbic acid, which was estimated as described by Roe and Kuether (1943).

Extraction and Estimation of Skin Histamine.—The piece of abdominal skin was freed from subcutaneous tissue before weighing. Each sample was ground in a mortar containing a little acid-washed sand, with

2 ml. N-HCl and 10 ml. distilled water for each g. of tissue. When sufficiently disintegrated it was transferred to an evaporating basin, the mortar and pestle being rinsed three times with normal saline. After boiling for 1 min. the extract was filtered through cotton-wool and the basin, funnel, and cotton-wool pad were rinsed twice with a little normal saline. The extract was brought to about *p*H 7.5 with N-NaOH and the volume adjusted by adding normal saline until 1 ml. was equivalent to 20 mg. tissue. The extract was stored in a refrigerator until assayed, within 6 hr. of preparation, on the atropinized guinea-pig ileum.

Demedullation of Adrenal Glands.—The adrenal glands of litter mate, male rats were demedullated within a week or two of weaning, using the technique described by Evans (1936). Anaesthesia was by intraperitoneal pentobarbitone (1 mg.), supplemented with ether. Upon recovery, each rat was given a few drops of 2% glucose solution by mouth from a teat pipette. The operated rats were kept in a room at 28° C. and given 0.2% saline to drink for three weeks following the operation. Regeneration of cortical tissue was sufficiently advanced by this time to enable the 0.2% saline to be replaced with water.

Depletion of Skin Histamine.—This was effected by a technique similar to that of Feldberg and Talesnik (1953). Intraperitoneal injections of 48/80 in normal saline were given, one in the morning and one at night for five days. On the first day, 1 μg./g. body weight was given at each injection. On the second day a total dose of 200 μg. was injected each time. On each successive day the dose was increased by 100 μg., until, on the fifth day, each rat received two injections, each of 500 μg. Observations were made on the third day following the last dose.

Results

Effect of Morphine Sulphate on Normal Rats.—Within 20 min. of the subcutaneous injection of morphine sulphate, normal rats became inactive and remained so for 2 to 3 hr. Their behaviour was in marked contrast to that of saline-injected controls. Morphine-treated rats showed gradually increasing activity between 3 and 5 hr. after the drug. Care was taken not to disturb them until they were taken from the cage, after the elapse of the appropriate time interval.

Between 1½ and 3 hr. after the injection most rats were indifferent to handling and remained lethargic ; a few were irritable and resented handling, although they remained immobile if left alone. These observations are consistent with those on other rodents in which high doses of morphine often cause reflex irritability without motor excitement.

The sequence of depression followed by a return to normal activity fitted in well with the pattern of events in the adrenal cortex. One and a half hours after the injection of morphine sulphate the ascorbic acid content of the glands was 70% of the control value. After 3 hr. the level was still low at 73%, but after 5 hr. it had returned to 98% of the resting level (Table I).

The abdominal skin histamine of these animals was estimated to determine the normal level so as to indicate the degree of depletion obtained in rats treated with compound 48/80. It was much higher in Hooded (average 48 μg. histamine base/g. of skin) than in Wistar rats (26 μg./g.). The mean values for abdominal skin histamine given in Table I are derived from both Hooded and Wistar stains.

Effect of Morphine Sulphate on Rats with Demedullated Adrenal Glands.—After demedullation, cortical tissue regenerates from the empty capsules to which a few cortical cells remain attached. In some rats only one gland regenerated ; in a few, one of the glands was very large and the other very small. No glands under 5.0 mg. were used in the experiments, as they seldom gave values comparable with those given by their larger partners. Adequate regeneration of the glands had usually occurred 28 days after operation, but the animals were not used until at least 40 days had elapsed.

The behaviour of the demedullated animals after morphine was similar to that of normal rats, but irritability seemed to be more frequent. There was a marked fall in body temperature. The drug was evidently more toxic to demedullated than to normal rats, as it often caused wheezy respiration, and two animals died between 2 and 5 hr. after injection.

One and a half hours after the injection of morphine the adrenal ascorbic acid had fallen to 75% and after 3 hr. stood at 73% of the control value. Five hours after the injection the level had risen to 89%. The fall in the ascorbic acid both at 1½ and at 3 hr. after morphine was significant ($P<0.001$), but at neither time was it significantly different from the fall in normal animals: at 1½ hr. $P>0.2$ and at 3 hr. $P>0.6$ (Table I).

The concentration of histamine in abdominal skin was appreciably lower in the demedullated rats than in the normal animals, so it was decided to investigate this phenomenon.

Behaviour of Skin Histamine in Normal and Demedullated Rats.—The rats which had low values for the abdominal skin histamine differed from those with higher values in two respects: they had demedullated adrenal glands and they were older. The difference might therefore be due either to age or to demedullation.

TABLE I
THE EFFECT OF MORPHINE SULPHATE ON THE ADRENAL ASCORBIC ACID OF RATS

	Drug (Morphine Sulphate 2 mg. or 0·9% Saline 0·1 ml. per 100 g. Body Wt.)	Duration of Action in Hr.	Ascorbic Acid Content (mg./100 g. Gland ± S.E.)	Ascorbic Acid as % Normal	No. of Glands	Abdominal Skin Histamine (μg. Base/g. Skin)
Normal	Saline	1–3	352±13	100	17	34
	Morphine	1·5	248±10	70	12	35
	,,	3·0	257±17	73	14	32
	,,	5·0	345±19	98	6	33
Demedullated	Saline	1–3	294±17	100	13	27
	Morphine	1·5	220± 8	75	17	25
	,,	3·0	213±10	73	12	24
	,,	5·0	263±14	89	5	31
Histamine depleted ..	Saline	1–3	409±15	100	13	8
	Morphine	1·5	324± 8	79	14	9
	,,	3·0	359± 8	88	12	11
	,,	5·0	367± 5	90	12	11
Demedullated and with low skin histamine	Saline	1–3	274± 9	100	10	10
	Morphine	1·5	221±13	81	8	13
	,,	3·0	252±13	92	10	13

To test these possibilities 48 six-week-old male Wistar rats (not litter mates) were divided into two groups. The adrenal glands of one group were demedullated; the other group had sham operations. Four animals from each group were taken eight days after the operation, and four more every fourth week for six months. The abdominal skin histamine was estimated; it fell gradually in both groups during this period.

In view of this, the abdominal skin histamine of one-day-old rats was determined; it was found to be lower than that of the six-week-old rats. It was concluded, therefore, that the abdominal skin histamine rises between birth and six weeks of age, and that the subsequent fall is a function of age and not of demedullation. These results are shown in Table II.

TABLE II
EFFECT OF AGE ON ABDOMINAL SKIN HISTAMINE OF WISTAR RATS WITH DEMEDULLATED ADRENALS AND NORMAL SHAM-OPERATED CONTROLS
(The animals were operated on when 42 days old. Each figure is the average from four animals)

Age (Days)	Histamine Base (μg./g. Abdominal Skin ± S.D.)	
	Demedullated Rats	Sham-operated Rats
1	—	18·7±1·8
50	27·2±6·8	25·7±5·2
70	23·1±2·6	23·3±4·7
98	17·1±5·1	20·4±2·9
126	14·3±2·3	13·1±4·0
154	11·5±3·5	16·2±2·9
182	10·9±3·0	8·5±1·2

Since Riley and West (1952 and 1953) have demonstrated a correlation between the amount of histamine and the mast cell content of certain tissues, skin sections were prepared at each stage of the experiment and stained according to their technique. These sections never showed more than eight mast cells in one high-power field, and since no gross changes were observed as the histamine content of the skin diminished with age, no conclusions could be drawn concerning the relationship between the skin histamine and the mast cell population.

Rats Having a Depleted Skin Histamine.—The technique used to deplete the tissue histamine was similar to that described by Feldberg and Talesnik (1953), who showed that treatment with 48/80 greatly reduced the histamine content of the skin, skeletal muscle, and heart, but not that of stomach, duodenum, and liver. In the present experiments, the skin histamine level was used as a guide to the degree of tissue histamine depletion. It was never reduced to undetectable amounts, but it was diminished to levels varying between 12 and 30% of normal.

The behaviour of the histamine-depleted rats after morphine sulphate was the same as that of similarly injected normal animals. At 1½ and 3 hr. the response of the adrenal ascorbic acid appeared to be less than in the normal animals. However, statistical analysis showed that this was not so: at 1½ hr. $P>0.4$ and at 3 hr. $P>0.1$ (Table I).

Rats with Demedullated Adrenal Glands and a Low Tissue Histamine.—The first two attempts to obtain rats with demedullated adrenal glands and a low tissue histamine failed. In the first, a group of rats was demedullated and, 36 days after the operation, treatment with 48/80 was begun. The animals proved very sensitive to the drug and were nearly all killed by the early doses. In the second attempt, a group of rats was first treated with 48/80 in gradually increasing doses as previously described. On completion of this treatment,

the rats were demedullated and given daily maintenance doses of 50 μg. of 48/80. Between 40 and 50 days after the operation the animals were taken for experiment, but were found to have a nearly normal level of abdominal skin histamine.

In view of these failures it was decided to determine the effect of morphine sulphate on the adrenal ascorbic acid of demedullated rats having a low tissue histamine in consequence of their age. Accordingly, a group of rats was demedullated and kept for 21 weeks after the operation before performing the experiment. In these animals the abdominal skin histamine was reduced to levels very nearly as low as those in animals pretreated with 48/80. The response of the adrenal ascorbic acid was less than in normal animals. The level was 81% of the normal figure 1½ hr. after the morphine sulphate and had returned to 92% 3 hr. after the injection. The difference between these falls and those seen in normal animals was significant: at 1½ hr. $P<0.05$ and at 3 hr. $P<0.01$.

Discussion

The fall in the rats' adrenal ascorbic acid caused by the subcutaneous injection of 2 mg./100 g. body weight of morphine sulphate is less in demedullated and in histamine-depleted rats than in normal animals, but the significance of the difference is very small, P never being less than 0.2. In rats having a low tissue histamine and demedullated adrenals the fall in adrenal ascorbic acid caused by morphine is significantly less than in normal animals ($P<0.05$ at 1½ hr. and $P<0.01$ at 3 hr.). It seems from these results that the effect of morphine sulphate on the rat adrenal cortex is affected partly by released histamine and partly by adrenaline released from the adrenal medulla. The fact that both the elimination of the adrenal medulla and the reduction of tissue histamine to very low levels is necessary to obtain a significant effect, suggests either that the individual part played by the released substances is very small, or that the one can compensate for the absence of the other. It must be emphasized that the liberation of histamine and adrenaline is probably not responsible for the whole effect on the adrenal ascorbic acid, since there is still a significant fall in demedullated, histamine-depleted rats. Porter (1952) has shown that intravenous adrenaline causes increased electrical activity in the posterior hypothalamus of the rat, and that destruction of this area prevents the eosinopenic response to the drug. Other stress stimuli had similar effects. It is possible, therefore, that the adrenaline and histamine released by morphine, as well as the morphine itself, may stimulate the hypothalamus.

It was observed that the abdominal skin histamine of the rat rises between birth and six weeks of age and then falls. This seemed to be inconsistent with the findings of Riley and West (1953) in man, the sow, and the cat: they reported a higher level of tissue histamine in adults than in infants. Their observations were based on determinations in infants aged from 1 to 7 days, and in fully grown members of the various species. It would be possible to present a similar picture for rats by selecting two appropriate points on the curve. The fall in the level of the rats' abdominal skin histamine with advancing age is incidentally apparent in the figures of Geiringer and Hardwick (1953), though they were not concerned with this particular phenomenon.

Summary

1. The subcutaneous injection of 2 mg./100 g. body weight of morphine sulphate caused a significant fall in the ascorbic acid content of rats' adrenals.

2. The same dose of morphine sulphate in rats with demedullated adrenals, and in animals with a depleted tissue histamine, caused a reduction in adrenal ascorbic acid; this reduction was not significantly less than that observed in normal rats.

3. The fall in adrenal ascorbic acid caused by a similar dose of morphine sulphate, in rats with demedullated adrenals plus depleted tissue histamine, was significantly less than that in normal rats.

4. The histamine content of the abdominal skin in Wistar rats was shown to increase between birth and six weeks of age; during the subsequent six months it diminished to a level considerably below that at birth.

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THE INTRACELLULAR DISTRIBUTION OF HISTAMINE IN DOG'S LIVER

BY

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Several years ago, working with portions of dog's liver which had been ground in saline and centrifuged, Trethewie (1938) demonstrated in the supernatant fluid cellular debris which was thrown down by further centrifugation. It contained histamine which could be brought into solution by heating. Feldberg and Kellaway (1938) were able to release histamine from particles found in the perfusate from dog's liver following the intraportal injection of staphylococcal toxin. The refined methods of tissue fractionation developed during the last ten years by Claude (1946), Hogeboom, Schneider, and Palade (1948) and other workers, have provided techniques enabling a further and more detailed investigation of this phenomenon. The recent demonstration by Blaschko and Welch (1953), that the pressor amines of adrenal medulla are for the most part held within particles thrown down in gravitational fields similar to those in which liver mitochondria are thrown down, suggested the possibility that histamine might be similarly localized in particles of defined size.

With these points in mind the histamine content of the particulate and non-particulate fractions obtained by differential centrifugation of sucrose homogenates of dog's liver tissue has been investigated.

METHODS

Liver tissue was obtained immediately after death from dogs which had been bled out under ether anaesthesia and the blood largely replaced by saline. Ten grams of tissue was cut into small pieces and then dispersed for 10 minutes in sucrose solution with a Potter–Elvehjem homogenizer. The volume was made up to 100 ml. The homogenate was centrifuged for 30 minutes at 900 *g* in order to remove cell nuclei, blood, and any tissue fragments which might have escaped disintegration. A portion of the resulting supernatant fluid (homogenate) was then centrifuged for 30 minutes at 22,000 *g*. The supernatant fluid was poured off and the sediment made up to the same volume with sucrose solution. In this way three fractions were obtained: the homogenate, the resuspended sediment, and the sediment-free supernatant fluid. In one experiment the resuspended sediment was centrifuged a second time at 22,000 *g* for 30 minutes in order to wash away any contaminating supernatant fluid, and the final sediment was resuspended in sucrose solution as before.

The sucrose solutions were usually 0.25 M or 0.88 M, as standard techniques for liver-cell fractionation have been devised using these particular media (Schneider and Hogeboom, 1951). In the later experiments 0.32 M-sucrose was used, which is isotonic with tissue fluids.

All manipulations were performed in the cold, using an ice-bath, and centrifugation was carried out in a refrigerated centrifuge at -1° C.

Assays were usually made on guinea-pig ileum suspended in oxygenated Locke's solution containing 6 mg./l. of hexamethonium bromide. Some samples were assayed by the depressor response produced in cats under chloralose anaesthesia. Before the assay the fractions were usually frozen and thawed in order to liberate any histamine from the particles, but in some experiments the samples were acidified immediately after their preparation and neutralized just before the assay.

RESULTS

Distribution of Histamine.—Altogether seven experiments were carried out in which dog liver homogenates were subjected to centrifugation and the fractions obtained assayed for their histamine content. The results of these experiments are shown in Table I, in which the histamine content of each fraction is expressed as micrograms of histamine dihydrochloride per gram of liver tissue. The histamine contents of the high speed supernatant fluid and sediment are also expressed as percentages of the histamine content of the homogenate from which they were derived.

In these experiments the histamine content of the homogenates varied from 8 to 23 micrograms (as dihydrochloride) per gram of fresh liver tissue. On centrifugation a fraction varying from 10–36% (mean 19%) was found in the supernatant fluid; from 52–100% (mean 76.5%) was found in the sediment. The histamine recovered in these

TABLE I

HISTAMINE CONTENT OF DOG LIVER HOMOGENATES AND OF THE FRACTIONS OBTAINED ON CENTRIFUGATION

Histamine in μg. dihydrochloride per g. liver. The histamine content of each fraction is also expressed as a percentage of the total histamine of the homogenate from which it was derived.

Expt. No.	Molarity of Suspending Medium	Method of Assay	Histamine Content of				
			Homogenate μg./g.	Supernatant Fluid μg./g.	Supernatant Fluid %	Sediment μg./g.	Sediment %
1	0·25	G.P.I.	10·6	3·8	36	7·5	72
2	0·88	G.P.I.	8	2	25	6	75
3	0·88	G.P.I.	23	1·5	6·5	20*	87
		C.B.P.	20	2	10	16*	80
4	0·25	G.P.I.	15	2·3	15	7·8	52
5	0·88	G.P.I.	19	3·5	18	19	100
		C.B.P.				20	
6	0·88	G.P.I.	39	12	31	27	69
7	0·32	C.B.P.	20	2·5	12·5 (Mean 19%)	15	75 (Mean 76·5%)

* The resuspended sediment was re-centrifuged and suspended in sucrose solution a second time.

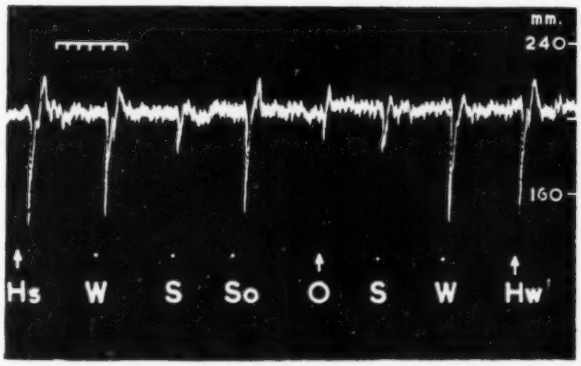

FIG. 1.—Blood pressure tracing from cat anaesthetized with chloralose, showing the effect of injecting the resuspended sediment in various media. Time, 30 sec. B.P. in mm. Hg. At Hs 1 μg. histamine dihydrochloride in 1 ml. isotonic sucrose; at W 0·5 ml. resuspended sediment made up to 1 ml. with distilled water; at S 0·5 ml. resuspended sediment made up to 1 ml. with isotonic sucrose; at So 0·5 ml. resuspended sediment made up to 1 ml. with isotonic sucrose with octylamine 10^{-3}: at O octylamine 10^{-3} in isotonic sucrose 1 ml.; at Hw 1μg. histamine dihydrochloride in 1 ml. distilled water.

two fractions varied from 67–118% of the amount in the original homogenate. There was no indication of a significant difference in the distribution of histamine when media of different tonicity were used.

The results given were obtained with fractions which had been either frozen and thawed or acidified and neutralized; however, when freshly prepared sediment was used another phenomenon was observed. When the particulate fraction, resuspended in isotonic sucrose, was further diluted with isotonic sucrose or isotonic saline solution before injection into the cat, either very little or no depressor effect was observed. On the other hand, when it was diluted with distilled water the injection was followed by a fall in blood pressure similar to that seen on injecting an equivalent quantity of acidified sediment (see Fig. 1).

When the particulate fraction was diluted with isotonic sucrose to which had been added *n*-octylamine, to give an octylamine concentration of 1 in 1,000, its injection produced the same fall in blood pressure as when it was diluted with distilled water. *n*-Octylamine in a concentration of 1 in 10,000 did not have this effect.

When the particulate fraction was diluted with isotonic sucrose to which had been added 48/80 (10 μg. in 1 ml.) its injection did not produce any greater fall in blood pressure than the slight fall which followed the injection of sediment alone in isotonic sucrose solution.

The depressor effects produced in the cat on intravenous injection of the standard histamine solution, and of the fractions obtained from dog liver, could not be obtained in a cat which had previously received 4 mg. of mepyramine.

DISCUSSION

Trethewie (1928) and Feldberg and Kellaway (1938) showed that a portion of the histamine in dog liver is contained in subcellular particles. In the experiments described in the present paper sucrose media were used. In the centrifugation procedure adopted the use of 0.25 M-sucrose results in the sedimentation of a "large granule" fraction frequently referred to as "mitochondria" and a "small granule" fraction frequently referred to as "microsomes" (Schneider and Hogeboom, 1951); with 0.88 M-sucrose only the large particles are thrown down. The lack of any marked difference in the histamine distribution between supernatant fluid and sediment, prepared in either medium, indicates that the histamine is principally in the large granule fraction. This is in agreement with Copenhaver, Nagler, and Goth (1953), who found that most of the histamine in liver is in the mitochondria.

Trethewie, and Feldberg and Kellaway, observed no fall of blood pressure on injecting the suspended particles intravenously into a cat, but were able to "release" the histamine by heating. It has been shown that the particles retain their histamine in isotonic sucrose or saline media. However, dilution of the sediment with distilled water causes a release of the histamine. It can also be released by *n*-octylamine in a concentration of 0.1%. This is the lowest concentration which Mongar and Schild (1953) found would cause the immediate release of histamine from guinea-pig lung. A lower concentration of *n*-octylamine failed to release histamine from dog-liver particles. 48/80

in a concentration of 0.01% did not cause the release of histamine from the particles; this concentration of 48/80 must have been about ten times greater than that which would be expected to obtain in the blood of cats, in which it caused a substantial fall in blood pressure (Paton, 1951).

It seems reasonable to infer that the liver particles remain intact in isotonic media so that any histamine they contain is incapable of producing its pharmacological effects; but that certain types of damage to the particles, such as freezing and thawing, acidification, heating, suspension in very hypotonic media or the addition of octylamine, cause the liberation of histamine into the media.

Summary

1. Homogenates of dog liver have been fractionated into particulate and non-particulate components by high speed centrifugation at low temperature.

2. Two-thirds or more of the histamine was found in the "large granule" fraction.

3. In isotonic media the particles retain their histamine so that its pharmacological activity is not apparent when the suspension is injected into cats.

4. The histamine can be released from the particles by a variety of procedures which might be expected to damage a surface membrane, and by the histamine releaser *n*-octylamine, but not by 48/80.

I wish to express my appreciation to Dr. H. Blaschko for his advice and help throughout the course of this work and to Professor J. H. Burn for allowing me to work in his Department during the tenure of a C. J. Martin fellowship of the National Health and Medical Research Council of Australia.

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THE MECHANISMS OF TOXICITY OF SOME IRON PREPARATIONS

BY

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Post-mortem and histological studies after the intravenous injection of toxic doses of iron preparations revealed extensive haemorrhages in the lungs (Nissim, 1949, 1953a, b). With some compounds the haemorrhages were present in the complete absence of precipitation, whereas with others their extent was much greater than would have been expected from the degree of precipitation produced. It seemed possible that the compounds concerned might possess an anticoagulant action which would account for this toxic manifestation. Coagulation tests were accordingly carried out on some iron compounds. The part played by capillary injury in producing haemorrhages was also investigated, as was the effect of iron preparations in the production of haemolysis *in vitro*.

METHODS

In vitro *Testing of the Anticoagulant Effect of Iron Compounds.*—In each of a series of 10 4-ml. test-tubes there was placed 0.32 ml. of a dilution, with physiological saline, of the iron solution to be tested: the range was from 2.0 to 0.004% Fe. A control tube was set up with 0.32 ml. of physiological saline only. A similar series of tubes containing 0.32 ml. of heparin in descending serial dilutions of 1.0 to 0.004% (100 i.u./mg.) was used for comparison. Fresh blood, obtained from the ear of a rabbit, was received into a small beaker and 1 ml. transferred quickly to each of the test-tubes. These were then shaken to ensure thorough mixing. The tubes, kept at room temperature, were observed at intervals for three hours and the clotting time in each tube was noted.

In vitro *Haemolysing Effect of Iron Compounds.*—A series of 10 test-tubes was set up and 1 ml. of the serially diluted iron solutions was pipetted into each (2–0.004% Fe). Rabbit blood was diluted a hundred times with physiological saline; 0.2 ml. of this diluted blood was added to each test-tube and the degree of haemolysis observed under the microscope.

RESULTS

Anticoagulant Effect.—"Ferrous chloride ascorbate" proved the most active of all the iron compounds studied (Table I), which is in striking agreement with the incidence of haemoptysis and massive pulmonary haemorrhages produced by it. This iron preparation had an anticoagulant activity which, by weight of its iron content, was almost equal to that of heparin, but the majority of compounds had an activity about 1/10 that of heparin. The least effective of all was saccharated iron oxide, which had only about 1/100 the activity of heparin.

TABLE I

THE ANTICOAGULANT EFFECT OF DIFFERENT IRON PREPARATIONS COMPARED WITH HEPARIN

1 ml. blood added to 0·32 ml. of solution of iron or heparin. Numerals represent coagulation time in min.

Conc. of Fe or Heparin %	Saccharated Iron Oxide. Sample G_4	Ferrivenin	Colloidal Ferric Hydroxide	Ferronascine Ferric Chloride Caramelate Ferrous Ascorbate (Alk. or Neutral)	Ferrous Chloride Pyruvate Iron and Ammonium Citrate Ferric Tartrate	Ferric Hydroxide Ferrous Ascorbate Ferric Chloride Lactate	Ferric Glucosate	Ferrous Chloride Ascorbate	Heparin
1	90	120	—	—	—	—	—	—	—
0·5	30	30	120	—	—	—	—	—	—
0·25	6	10	30	—	—	—	—	—	—
0·125	5	6	15	90	120	—	—	—	—
0·0625	5	5	10	15–60	30–60	120	—	—	—
0·0312	5	5	5	5–30	5–30	30	120	—	—
0·0160	5	5	5	5–10	5–10	15	30	120	180
0·0080	5	5	5	5	5	5	5	30	90

Haemolysing Effect.—The haemolysing effect of four iron compounds was studied—saccharated iron oxide, ferric glucosate, "ferric hydroxide ferrous ascorbate" and iron and ammonium citrate. The results are shown in Table II, and reveal no striking differences amongst the compounds. The haemolysing effect disappeared quickly with dilutions below 1–0.5% Fe, and must therefore be negligible *in vivo*, as iron injected intravenously is very rapidly diluted in the blood stream.

TABLE II

THE HAEMOLYSING EFFECT OF IRON PREPARATIONS

0.2 ml. of 1% blood added to 1 ml. of serially diluted iron solution

Percentage Concentration	Saccharated Iron Oxide	Ferric Glucosate	Ferric Hydroxide Ferrous Ascorbate	Iron and Ammonium Citrate
2·0	Completely haemolysed	Completely haemolysed	90% haemolysed	90% haemolysed
1·0	90% haemolysed	"	Severely crenated cells	Severely crenated cells
0·5	10% haemolysed	90% haemolysed	"	"
0·25	Severely crenated cells	10% haemolysed	"	"
0·125	" "	Severely crenated cells	"	"
0·0625	" "	"	Some crenation	Some crenation
0·03125	" "	"	"	"
0·016	Some crenation	"	"	"
0·008	" "	Some crenation	"	"
0·004	" "	"	"	"

Iron compounds were not found to have any agglutinating effect on the red cells.

Capillary Damaging Effect.—(*a*) *Saccharated Iron Oxide and Heparin.* To find to what extent this anticoagulant effect is responsible for the pulmonary haemorrhages, saccharated iron oxide was given together with heparin, to see whether it would produce massive haemorrhages comparable with those seen after ferric glucosate. The anticoagulant activity of ferric glucosate is about half that of heparin.

Two rabbits were therefore injected intravenously with 22.5 and 45 mg. Fe/kg. of saccharated iron oxide (G_4), mixed with 11.25 and 22.5 mg. heparin/kg. respectively. It is generally accepted (Wilson and Schild, 1952) that the action of heparin is maintained for 3–4 hours, during which time the haemorrhagic manifestations of ferric glucosate become evident. The two rabbits were killed 24 hours after the injection; the first showed no haemorrhages whatever, whereas the second revealed only a few punctate haemorrhages in the lungs—in contrast with the massive lesions seen with ferric glucosate, and which cause death in 2–6 hours. Hence, the anticoagulant effect presumably cannot, by itself, account for the pulmonary haemorrhages which ferric glucosate causes.

(*b*) *Pulmonary Oedema after Different Iron Preparations.* Pulmonary oedema and pleural effusion followed the administration of some iron preparations. They were the predominant toxic features after intravenous "ferric chloride lactate," some of the rabbits spouting oedema fluid even as the iron preparation was being injected. Pulmonary oedema was also marked after "ferric hydroxide ferrous ascorbate" and ferric glucosate, and to a lesser extent after some other iron compounds, such as ferric tartrate, iron and ammonium citrate, and "Ferronascine" (Roche). The injury to the capillary endothelium, as well as to the epithelial lining of the alveoli, occurred in three stages. In the first stage there was shortening and thickening of the cells of the alveolar walls, resulting in semi-collapse of the alveolar air sacs. In the second stage oedema fluid collected in the alveoli, distending them again, and giving the lungs a more solid consistency. In the third stage oedema fluid appeared in the pleural cavities.

DISCUSSION

The experiments reported in this paper have uncovered two major factors in the toxicity of some iron compounds. A definite and measurable anticoagulant activity has been demonstrated for each of the compounds studied. This activity is particularly striking with "ferrous chloride ascorbate" and ferric glucosate, the two iron preparations responsible for the severest haemorrhages in the lungs. By administering saccharated iron oxide with heparin, however, it was shown that anticoagulant activity alone was not sufficient to explain massive haemorrhages in the lungs. The considerable pulmonary oedema associated with the haemorrhages of ferric glucosate indicated a severe degree of capillary damage as well. These experiments lead to the conclusion that capillary damage plays an important role in the production of haemorrhage by such iron compounds. When capillary injury is not associated with marked anticoagulant effect, pulmonary oedema and pleural effusion without haemorrhage result. The present experiments provide a further explanation for the very low toxicity of saccharated iron oxide, which appears to be devoid of any capillary damaging effect in addition to being the most feebly anticoagulant of the iron preparations investigated.

SUMMARY

1. Pulmonary haemorrhages produced by iron compounds without evidence of iron precipitation led to a study of their anticoagulant activity.

2. One compound, " ferrous chloride ascorbate," shows an anticoagulant activity (by weight of iron) almost as high as that of heparin.

3. Pulmonary oedema produced by some iron preparations suggests severe capillary damage; when this is associated with a high anticoagulant activity of the compound the effects are much more severe.

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ANALGESIC AND OTHER ACTIONS OF SOME DITHIENYLBUTENYLAMINE COMPOUNDS IN MAN

BY

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Adamson (1950) synthesized some dithienylalkenylamines which had atropine-like, antihistaminic and local anaesthetic properties. In addition, it was found that these compounds were powerful analgesics in animals (Adamson and Green, 1950; Green, 1953). Since they have a different chemical structure from other drugs which produce a morphine-like action it was obviously important to study their actions in man.

In this paper we describe such an investigation which has been divided into two parts, the first dealing with observations on the effects of these drugs in normal subjects, and the second describing the results of a pilot trial in patients.

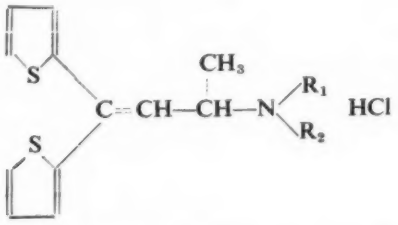
The observations on normal persons have been designed mainly to study side-effects of the drugs, which can be more accurately recorded in this way than in a clinical trial (Denton and Beecher, 1949). The effects on respiration have been fully investigated.

Previous studies had shown that the dithienyl compounds were less potent than morphine, but rather more potent than pethidine in relieving experimental ischaemic muscular pain (Keele, 1952). However, we agree with Beecher (1951) that a clinical trial is the only satisfactory way of estimating the analgesic potency of a drug, so as soon as possible we began to make observations on patients.

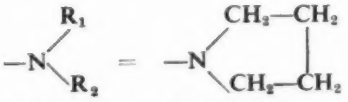
Owing to the occurrence of certain side-effects in normal subjects we had to make these studies more of a "pilot trial" than a controlled investigation. Nevertheless, we have recorded results which, in our opinion, justify definite conclusions concerning the therapeutic value of these dithienyl compounds.

METHODS

Drugs Used.—(*a*) The general formula of the dithienylbutenylamine series is:

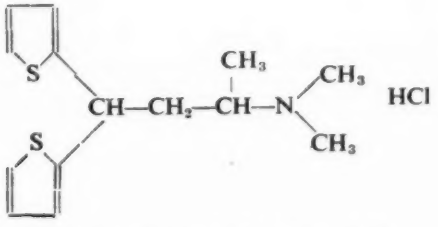


The compounds used were: 1C50, where R_1 is CH_3 and R_2 is C_2H_5; 191C49, where R_1 and R_2 are both C_2H_5; and 268C49, where



(Adamson & Green, 1950)

The following dithienylbutylamine compound, 489C49, was also used:



(Adamson, Duffin, and Green, 1951)

Compound 489C49 is readily soluble in water; the solution can be sterilized by autoclaving, and is stable for several months. It was made up as a solution containing 100 mg. in 1-ml. ampoules. Compound 268C49 has similar physical properties and was made up in a solution containing 75 mg./ml.

Compounds 191C49 and 1C50 are unstable in aqueous solution, and were available as granular white powders (50 mg. in a glass phial); the powder was easily dissolved in 1 ml. of sterile water before injection. (*b*) Other analgesic drugs used were: morphine sulphate, pethidine hydrochloride, and (±)- and (−)-methadone hydrochloride.

All the drugs were injected intramuscularly (into the deltoid) in both normal volunteers and in patients. The dithienyl compounds are inactive by mouth.

Studies in Normal Volunteers

The drugs were administered to 9 male and to 3 female student volunteers, aged 22 to 29 years, in order to record the degree of respiratory depression and the occurrence of side effects. The effect on breathing was measured by recording the amplitude and rate of respiration.

On the day of an experiment the subjects had a normal breakfast at 8 a.m., after which they took no more food till the experiment was over. They came to the B.M.R. Department of the Courtauld Institute of Biochemistry at about 1 p.m. and lay on couches for ½ hr. before the first respiratory record was made. Breathing was graphically recorded by the Benedict-Roth closed circuit apparatus; the subject breathed oxygen, and the CO_2 was absorbed by soda lime. Each subject used the same individual machine throughout these studies. Each respiratory record was taken for 7 min., the last 5-min. period being used for measurements of the rate and amplitude of breathing, from which the pulmonary ventilation was calculated.

Preliminary respiratory records were made after ½ hr. and after 1 hr. of rest on the couch. The drug or control solution was then injected and further respiratory records were made ½, 1, 2, 3, and 4 hr. after the injection. In all these experiments the nature of the injected solution was unknown to the recipient, and on many occasions it was also unknown to the administrator. The occurrence of side effects was noted, and arterial blood pressure and heart rate were recorded during most of the experiments.

Estimation of Respiratory Depression

In calculating the effect of a drug on respiration the second of the two preliminary records (after 1 hr. rest) was taken as the normal standard of reference, and subsequent changes in respiratory rate and amplitude, and in pulmonary ventilation, were expressed as percentage deviations from these standard values.

The total respiratory depression (or stimulation) after injection of the drug was calculated from the percentage deviations plotted against time. The total effect was proportional to the area under the curve expressed in arbitrary units (see Fig. 4).

Methods in Clinical Trial

The clinical trial was performed almost exclusively on patients with post-operative pain, the patients being selected according to the following criteria:

1. The pain had to be of sufficient intensity for the patient to require a powerful analgesic drug.

2. The post-operative condition of the patient had to be good enough to justify the administration of a new drug whose side effects were not well known.

3. The patients had to be sufficiently intelligent, and sufficiently recovered from the effects of the anaesthetic drugs, to understand and answer simple questions concerning their feelings.

4. Patients who had had abdominal operations were generally excluded from the trial, since the pain associated with spasm or distension of the gut is variable in its time-course, and can be relieved by measures which lead to the passage of flatus.

The patients were, of course, unaware of the nature of the drug which they were given, and did not know that they were participating in a clinical trial. We should also have preferred the administrator to be ignorant of the nature of the drug which he injected, as in the investigation described by Keats, Beecher, and Mosteller (1950). However, in our trial we were dealing, not with well-tried drugs of known toxicity, but with new compounds which had to be suspected of undesirable properties. We considered that it was not justifiable to give such compounds as "unknowns" to patients just recovering from operations. Other factors concerned in the administration and assessment of the drugs were dealt with as follows:

All drugs were given intramuscularly into the deltoid by one of us (P. F.) who also made all the observations on the effects. In recording the time-course of the pain it was not possible to use pain charts made by the patient as described by Hewer, Keele, Keele, and Nathan (1949) in their studies on the effects of analgesic drugs in patients with chronic pain. Instead, the patients were carefully questioned at frequent intervals concerning the site and intensity of pain, and notes were made on their general appearance, and on the presence of restlessness or anxiety. In recording the intensity of pain the observer questioned each patient so as to grade the pain on the following scale: none=0, slight=1, moderate=2, severe=3, very severe=4. In many cases it was obvious from general appearances, even before questioning the patient, that there had, or had not, been any relief of pain. The intensity of pain was recorded before injection of a drug, ½ hr. after the injection and then at hourly intervals until another injection was required, or until it appeared that the pain was not likely to recur. In addition to the usual data recorded on case sheets, particular attention was paid to the occurrence of side effects, and arterial blood pressure, heart rate and respiratory rate were recorded when the patient was questioned about his pain. It quite often happened that a patient was asleep when the time for questioning arrived; pain was then assumed to be absent and the patient was allowed to sleep on.

The new drugs of the dithienylbutenylamine series were given as first injections as often as possible, in

order to obtain a sufficiently large number of observations on their action. Even where only one injection was given it was useful to know that the drug had apparently been effective, the more so since the number of patients who require analgesic drugs for post-operative pain is relatively small. Of course, much more valuable information was obtained when several injections of analgesic drugs were needed, so that the effects of a new drug could be compared with those of morphine and pethidine.

We did not think it was justifiable to use injections of saline as controls, since, as described by Beecher (1951), they give satisfactory pain relief in no more than 20% of patients. What we did to test our patients' powers of discrimination was to see if they could distinguish the effects on their pain of two different doses of the analgesic drugs. In some instances the small doses were quite ineffective, which was more valuable information than would have been provided even by injections of saline, as it gave us the lower limit of effective dosage. This procedure was particularly valuable in our studies of compound 1C50.

In addition to pethidine hydrochloride and morphine sulphate, the following dithienyl drugs were used in the clinical trial: 268C49; 489C49; 1C50; a few injections of 191C49 were also given.

RESULTS

Studies in Normal Subjects

Actions of Drugs on Respiration

Effect of the Spirometer on the Rate of Breathing.—Early in the course of the breathing experiments it was noted that the respiratory rate at rest was reduced when the subjects were made to breathe into the spirometer. In 50 experiments the rate was counted just before the machine was connected, and again from the subsequent graphic record. Table I shows the effect of the spirometer on the rate of breathing in two subjects who received 50 mg. pethidine and 25 mg. 1C50 respectively. The rate of breathing is seen to be slower when the subject is "on the machine," and analysis of the 50 records showed that connexion to the spirometer reduced the respiratory rate by an average of 21%. This change is probably due to the resistance presented by the valves in the machine, and the amplitude of breathing is presumably increased to compensate for the reduction in rate.

TABLE I

EFFECTS OF PETHIDINE AND COMPOUND 1C50 ON THE RATE OF BREATHING (BREATHS PER 5 MIN.) IMMEDIATELY BEFORE AND AFTER CONNECTING THE SUBJECT TO THE BENEDICT-ROTH SPIROMETER

Drug, Dose, and Subject		Time after Injection (Hr.)					
		0	½	1	2	3	4
Pethidine HCl 50 mg. (subject, L.P.)	Before	70	80	70	70	80	70
	After	60	61	60	56	54	57
Compound 1C50, 25 mg. (subject, E.G.)	Before	75	60	50	50	70	65
	After	57	30	37	41	45	58

Effect of Drugs on Breathing.—The effects of the drugs on breathing were studied in 250 experiments on 12 normal volunteers.

Typical effects of two of the drugs are seen in Figs. 1 and 2, which show the two different ways in which respiratory depression may be revealed. Fig. 1 shows the response of subject L.P. to 25 mg. 1C50. It will be noted that the amplitude of respiration was much reduced (mean tidal volume lowered from 580 ml. to 330 ml.), the rate of breathing being unchanged. On the other hand, as shown in Fig. 2, the response in subject E.G. to 5 mg. (–)-methadone was characterized by slowing

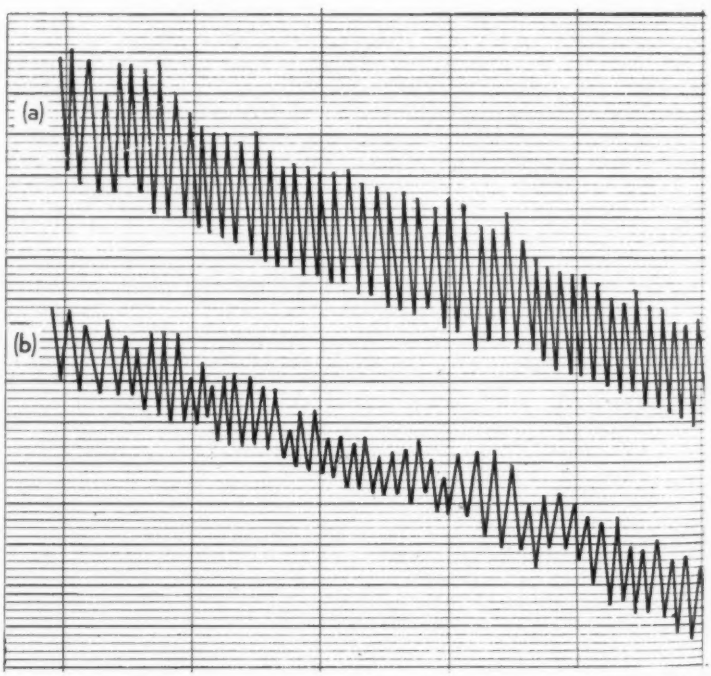


FIG. 1.—Effect of compound 1C50 on spirometer record of breathing. Subject L.P., ♂ (*a*) record taken before drug; (*b*) record taken ½ hr. after intramuscular injection of 25 mg. 1C50. In (*a*) respiratory rate, 46/5 min.; mean tidal volume 580 ml.; pulmonary ventilation in 5 min., 26.68 l. In (*b*) respiratory rate, 45/5 min.; mean tidal volume, 330 ml.; pulmonary ventilation in 5 min., 14.85 l.

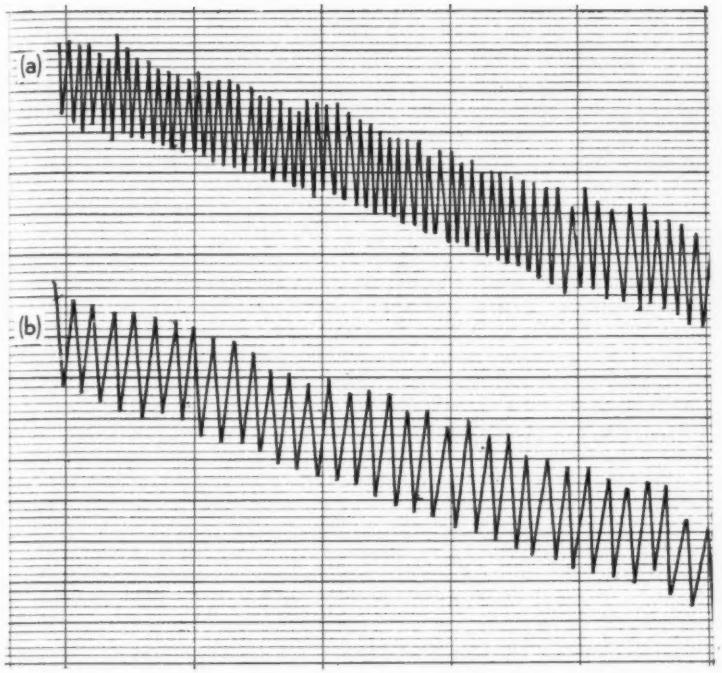


FIG. 2.—Effect of (–)-methadone on spirometer record of breathing. Subject E.G., ♀ (*a*) record taken before drug; (*b*) record taken 1 hr. after injection of 5 mg. (–)-methadone hydrochloride. In (*a*) respiratory rate, 59/5 min.; mean tidal volume, 450 ml.; pulmonary ventilation in 5 min., 26.55 l. In (*b*) respiratory rate, 32/5 min.; mean tidal volume, 510 ml.; pulmonary ventilation in 5 min., 16.32 l.

from 59 to 32 breaths/5 min., and the mean tidal volume was actually increased from 450 ml. to 510 ml.

Fig. 3 shows a typical response to a saline injection in subject H.C. There was no significant change in the character of the respiratory record after the injection.

In assessing the effects of these drugs we have calculated the pulmonary ventilation during the last 5 min. of each period of recording. In the experiment illustrated in Fig. 1 the pulmonary ventilation was reduced from 26.68 to 14.85 l./5 min. In the experiment shown in Fig. 2 the pulmonary ventilation was reduced from 26.55 to 16.32 l./5 min. After saline (Fig. 3) the pulmonary ventilation fell from 26.22 to 25.49 l./5 min.

The total respiratory changes during the experimental period are illustrated in Fig. 4, which shows the areas obtained in subject E.G. after injections of saline (4*a*) and 25 mg. of compound 1C50 (4*b*). After saline the area was −6.7, and after 1C50 it was −49.5 arbitrary units (see Method for explanation). In this way the effects of various injections were summarized numerically, and comparisons of the activity of different compounds could be made by standard statistical treatment of the area values.

The results obtained in 12 subjects after injection of saline, 25 mg. of 268C49, 25 mg. of 489C49, 50 mg. of pethidine, 10 mg. of morphine and 25 mg. of 1C50 respectively, are presented in Fig. 5, in which the means of the areas of percentage deviations, with 95% fiducial limits, are shown graphically. The results are set out to show the order of increasing depression of breathing from saline, which caused no depression, to compound 1C50, whose effect was greater than that of morphine.

A statistical analysis of the effects of these injections was made. The results are presented in Table II, which shows the values for "t" and the levels of significance for comparison of the means of the injections with the mean of saline. Table III shows the

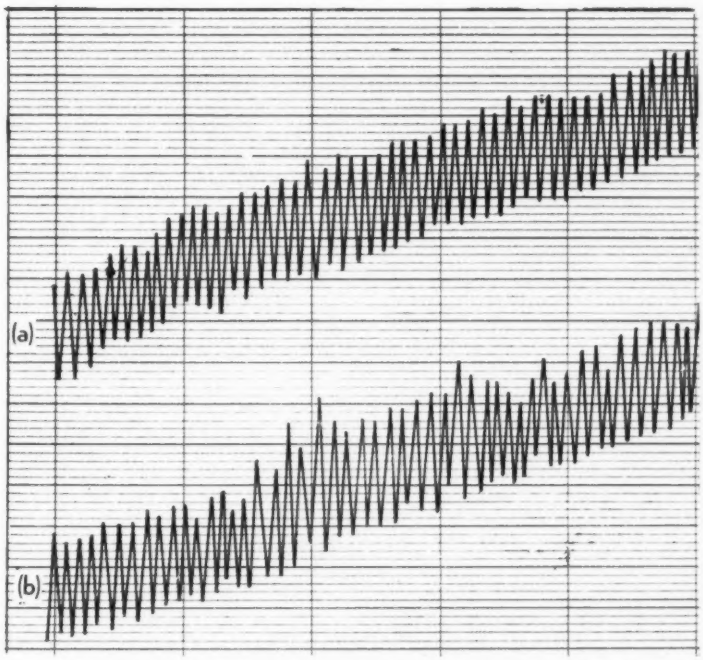


FIG. 3.—Effect of saline on spirometer record of breathing. Subject H.C., ♂ (*a*) before injection; (*b*) 1 hr. after injection of 1 ml. 0.9% NaCl solution. In (*a*) respiratory rate, 49/5 min.; mean tidal volume, 535 ml.; pulmonary ventilation in 5 min., 26.22 l. In (*b*) respiratory rate, 48/5 min.; mean tidal volume, 531 ml.; pulmonary ventilation in 5 min., 25.49 l.

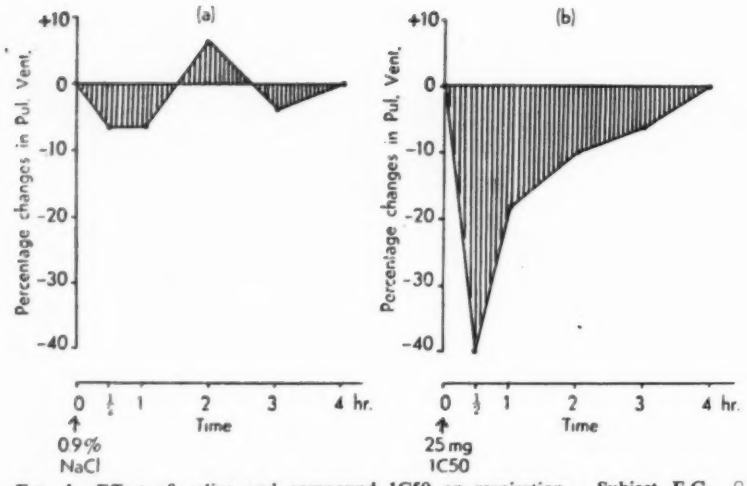


FIG. 4.—Effect of saline and compound 1C50 on respiration. Subject E.G., ♀. Ordinates: percentage changes in pulmonary ventilation, the zero value being the 2nd pre-injection reading. Abscissae: time in hours. (*a*) Injection of saline. The area under the curve is −6.7 arbitrary units (see Methods). (*b*) Injection of 25 mg. 1C50 i.m. The area under the curve is −49.5 arbitrary units.

significant differences found between the means of some of the injections. These data confirm the order of respiratory depressant potency seen graphically in Fig. 5.

TABLE II

STATISTICAL ANALYSIS OF THE EFFECTS OF THE VARIOUS INJECTIONS ON RESPIRATION. THE RESULTS ARE ARRANGED DOWNWARDS IN ORDER OF INCREASING EFFECT

Injection	No. of Readings	Mean of Area of % Deviation	Comparison of Means of Injections with Mean of Saline		
			t-value	Degrees of Freedom	Level of Significance
Saline	28	+1·2	—	—	—
268C49, 25 mg.	16	−18·1	1·96	42	—
489C49, 25 mg.	18	−27·1	2·94	44	1%
Pethidine, 50 mg.	17	−33·0	3·54	43	0·1%
Morphine, 10 mg.	17	−40·5	3·96	43	0·1%
1C50, 25 mg. ..	20	−50·3	5·77	46	0·1%

TABLE III

COMPARISON OF RESPIRATORY DEPRESSANT ACTIONS

To show significant differences found between means of drug injections (see Fig. 5 and Table II). (The remaining comparisons of groups showed no significant differences)

Between Groups	t-value	Degrees of Freedom	Level of Significance
268C49 (25 mg.) and morphine (10 mg.) ..	2·36	31	5%
268C49 (25 mg.) and 1C50 (25 mg.) ..	4·50	34	0·1%
489C49 (25 mg.) and 1C50 (25 mg.) ..	3·18	36	1%
Pethidine (50 mg.) and 1C50 (25 mg.) ..	2·42	35	2%

Effect of N-Allylnormorphine on Respiratory Depression by 1C50.—The effect of N-allylnormorphine (nalorphine) on the respiratory depression produced by compound 1C50 was studied in three subjects. A typical result is seen in Fig. 6, which shows the effect of intravenous injection of 10 mg. nalorphine on the spirometer record of subject H.C. when his breathing had been previously depressed by injection of 25 mg. of 1C50. Both rate and depth of breathing were stimulated within 1 min. of the injection.

Effects of the Various Drugs on Blood Pressure and Heart Rate.—Although there were frequent reductions in blood pressure (e.g. 10–15 mm. Hg systolic and 5–10 mm. Hg diastolic) and in heart rate (e.g. 10–15 beats/min.), after injections of the drugs, these changes were not consistently greater than those seen after saline, and will not be further analysed.

Other Effects

The symptoms and signs produced by the various injections in all 12 subjects are listed in Table IV. *Drowsiness* was the only symptom which followed

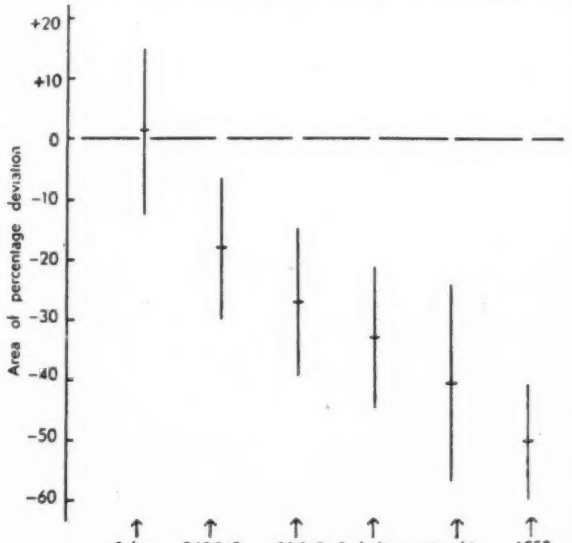


FIG. 5.—Summarized effects of drugs on respiration. The graph shows the means, with 95% fiducial limits, of the areas of percentage deviations in all subjects who received the substances indicated. The substances have been arranged in order of increasing potency of respiratory depression from saline through compound 1C50. See Table II for further details and analysis of these results.

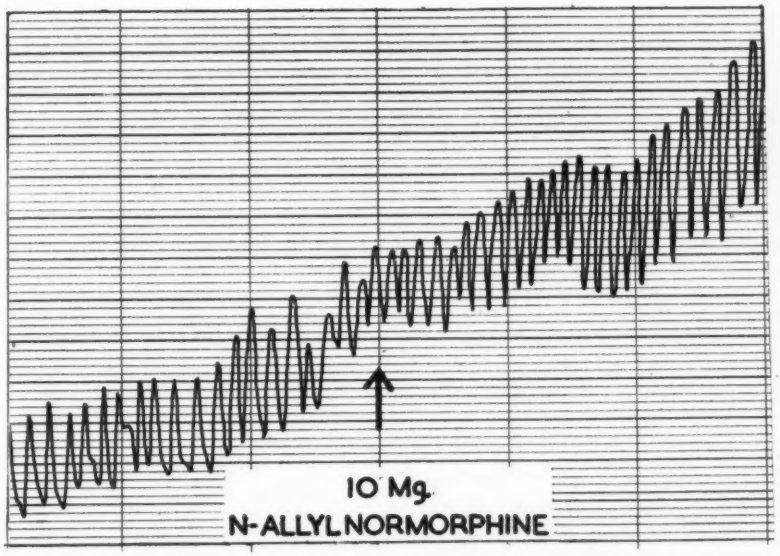


FIG. 6.—Effect of intravenous N-allylnormorphine on respiratory depression from compound 1C50. Spirometer record from subject H.C., ♂. Tracing to left of arrow shows breathing depressed by prior injection of 25 mg. 1C50. At arrow, 10 mg. N-allylnormorphine was injected i.v. Note rapid onset of respiratory stimulation.

injection of saline (5 out of 37 occasions) and it was only recorded when the subject was under the impression that he had received an active drug. With the drugs, drowsiness occurred almost invariably, and *sleep* quite frequently, especially after 489C49 and 1C50.

TABLE IV

SIDE-EFFECTS OF DITHIENYL COMPOUNDS IN 12 NORMAL SUBJECTS

The numerals in brackets at the head of each column indicate the total number of injections)

Side-effect	Drugs and Doses						
	Saline	Morphine SO_4 10 mg.	Pethidine HCl 50 mg.	489C49 25 mg.	268C49 25 mg.	1C50 25 mg.	(−)-methadone 5 mg.
	(37)	(29)	(28)	(19)	(18)	(23)	(13)
Drowsiness ..	5	21	23	19	14	21	9
Sleep	—	7	9	15	6	12	—
Uneasy stomach	—	6	—	2	—	—	1
Nausea ..	—	16	3	2	1	1	2
Vomiting ..	—	2	—	—	—	—	1
Pallor and sweating	—	7	—	1	—	—	1
Faintness ..	—	4	—	—	—	—	—
Ataxia	—	5	—	2	—	—	1
Light-headedness	—	—	2	4	1	2	2
"Drunkenness"	—	1	—	5	—	—	1
Euphoria ..	—	7	10	5	3	11	6
Headache ..	—	3	1	—	—	—	—
Photophobia ..	—	—	—	8	—	1	—
Dizziness ..	—	2	1	4	1	9	1
Difficulty in focusing ..	—	3	2	3	—	3	1
Mistiness of vision ..	—	—	—	8	1	4	—
Warm glow ..	—	5	7	8	2	8	1
Itching ..	—	—	1	—	—	5	—
Dry mouth ..	—	5	7	8	2	8	1
Muscular weakness	—	—	—	9	1	4	—

Table IV also shows that *nausea* was much more frequent with morphine than with any of the other drugs, the dithienylbutenylamine compounds being particularly free from this complication. After morphine the syndrome of nausea, pallor, sweating, faintness, and occasionally *vomiting* was most liable to occur when the subjects stood up and walked about at the end of the experiment.

The occurrence of *euphoria* with the new compounds was frequent enough to show that these drugs must be regarded as possible drugs of addiction.

Among the other symptoms, *dizziness* occurred most frequently with the dithienyl compounds, particularly 1C50. *Mistiness of vision*, also most marked with the new substances, was usually described as an appearance of "smoke moving up the wall." Photophobia was most frequent with 489C49.

A most important side action was the development of *muscular weakness*, most often with 489C49, but also recorded after 1C50 and 268C49. It did not occur after morphine, pethidine or (−)-methadone. The weakness was widespread but was greatest in the hands and arms, and in one subject (H.C.) the facial muscles were so greatly affected as to give a "myasthenic facies." This muscular weakness appeared to be idiosyncratic, since it did not occur in most subjects even when larger doses than those used here were given. The symptom was very unpleasant to those who experienced it (2 out of 12 subjects), and its occurrence led us to be very cautious in making our initial therapeutic invesigations on the analgesic actions of these drugs in patients. The development of muscular weakness in patients after 489C49 will be described in the next part of this paper.

Dr. G. A. Mogey, when at the Wellcome Research Laboratories, studied the actions of 489C49 on the cat's sciatic nerve–gastrocnemius preparation and on the rat's phrenic nerve–diaphragm preparation. In the cat the drug produced no signs of neuromuscular block, but in the rat diaphragm there was some evidence of direct depression of the muscle fibre with concentrations exceeding 1.0 mg./100 ml. Smaller concentrations (0.2 mg./100 ml.) reduced the size of repetitive

responses to long stimuli (5.70 msec.) and potentiated the paralysis produced by (+)-tubocurarine.

Clinical Trial

Illustrative Records

Table V shows the effects of 1C50, pethidine, morphine and 396C50 (a new methadone derivative) in a patient with post-operative gangrene of the foot. It will be noted that 50 mg. of compound 1C50 had no effect on two occasions; on one other occasion this dose was effective when given 1 hr. after a previous dose of 50 mg.; 100 mg. of 1C50 was effective on five occasions. Compound 396C50 in a dose of 5 mg. had no effect.

TABLE V

ACTIONS OF VARIOUS DRUGS, IN ORDER OF THEIR ADMINISTRATION, ON PAIN ASSOCIATED WITH GANGRENE OF FOOT FOLLOWING ARTHRODESIS IN PATIENT R.A. (♂, 50)

Drug and Dose	Pain*		Time (Hr)	
	Before	After	Peak	Duration
1C50, 50 mg.	3	3	—	0
1C50, 50 ,, (1 hr. later)	3	1	½	6+
1C50, 50 ,, ...	4	4	—	0
Pethidine HCl, 100 mg. ..	4	0 (s)	—	5+
1C50, 100 mg.	3	0	½	4½
1C50, 100 ,, ...	3	0 (s)	½ (s)	6½
1C50, 100 ,, ...	3	0 (s)	½ (s)	8½+
Morphine SO_4, 10 mg. ..	3	1	½	4½
1C50, 100 mg.	3	1	½	5½
†396C50, 5 mg.	3	3	—	0
1C50, 100 mg. (1½ hr. later) ..	3	0 (s)	½ (s)	6½+

* Scale of pain: 0=no pain, 1=slight pain, 2=moderate pain, 3=severe pain, 4=very severe pain, 0 (s)=sleep.

† Compound 396C50 is the methylethylamino-analogue of methadone.

The + sign after duration figures means that end-point was not observed.

In Table VI are shown the results of a number of injections in a patient with secondary deposits in the spine associated with carcinoma of the breast (the patient was having radiotherapy). In this Table the results have been grouped according to the drugs used and not in the order of injection. Morphine "X" was a microcrystalline preparation which was supposed to be long acting. Injections of 32 and 50 mg. of this preparation were quite ineffective, suggesting that it was not absorbed in adequate amounts. Morphine mucate (32 mg.) has a longer action than morphine sulphate (16 mg.) but it is impossible to say whether this difference is due to the difference in dosage of morphine or to slower absorption of the mucate. Pethidine (100 mg.) appeared to be the weakest and shortest lasting of the effective drugs. Compound 1C50 had a particularly marked hypnotic action in this patient. We should like to emphasize that the small or negative responses to 50 mg. of 1C50 and to 5 mg. of 396C50 in Table V, and to 32 and 50 mg. of morphine "X" in Table VI, serve as controls for the positive effects of the other injections.

TABLE VI

ACTIONS OF DRUGS ON PAIN DUE TO SECONDARY DEPOSITS IN SPINE IN A PATIENT (G.N., ♀, 56) WITH CARCINOMA OF THE BREAST

Pain scale as in Table V

Drug and Dose	Pain		Time (Hr.)	
	Before	After	Peak	Duration
*Morphine "X," 32 mg. ..	3	4	—	0
50 ,, ..	3	3	—	0
Morphine mucate, 32 mg. ..	3	1	1½	8½
	3	0 (s)	½ (s)	7½
	3	1	3	8
Morphine SO_4, 16 mg. ..	3	1	½	4½
	4	0 (s)	1½ (s)	4½
Pethidine HCl, 100 mg. ..	3	2	1½	4½
	3	2	½	2
	4	1	½	3
1C50, 100 mg.	3	0 (s)	1 (s)	7½
	4	0 (s)	½ (s)	9
	4	0 (s)	½ (s)	21+
	3	0 (s)	½ (s)	5

* Microcrystalline preparation of morphine.

Relief of Pain After 268C49

This drug was given in doses of 75 and 100 mg. and was clearly a less effective analgesic than 489C49 and 1C50. On 13 out of 26 occasions injection of 268C49 did not produce satisfactory relief of pain.

Relief of Pain After 489C49

This drug produced complete relief of pain in all of 10 injections (50 mg.). However, the side-effects, especially the muscular weakness which will be discussed later, led us to abandon further trials of this otherwise promising drug.

Relief of Pain After 1C50

In view of the relative ineffectiveness of 268C49 and the toxicity of 489C49, we decided to restrict our further investigations to 1C50. The results are set out in Table VII. We have assessed the effectiveness of 50 and 100 mg. of 1C50 by comparison with 100 mg. of pethidine hydrochloride and 10 mg. of morphine sulphate on the relief of severe or very severe pain and have classified the results under the headings "complete relief" and "satisfactory relief."

The first point which Table VII reveals is the capacity of the patients to discriminate between the effects of 50 and 100 mg. of 1C50. This is most strikingly shown by comparing the percentage of occasions on which complete relief of very severe or severe pain occurred, following the two doses of this drug. With 50 mg. complete relief occurred after 26 (38%) out of 69 injections. With

TABLE VII

RELIEF OF SEVERE OR VERY SEVERE PAIN BY COMPOUND 1C50, PETHIDINE, AND MORPHINE

(Satisfactory relief = pain reduced to slight or zero intensity)

Drug and Dose	Pain Before Drug Administration	Total No. of Drug Administrations (Figures in brackets show No. of Patients)	Administrations giving Complete Relief	Administrations giving Satisfactory Relief
1C50, 50 mg.	Very severe	22 (11)	5	12
	Severe	47 (26)	21	33
		69	26 (38%)	45 (65%)
1C50, 100 mg.	Very severe	14 (8)	7	13
	Severe	70 (37)	56	69
		84	63 (75%)	82 (98%)
Pethidine HCl, 100 mg.	Very severe	8 (6)	3	3
	Severe	48 (31)	24	39
		56	27 (48%)	42 (75%)
Morphine SO_4 10 mg.	Very severe	11 (6)	6	9
	Severe	25 (21)	18	25
		36	24 (67%)	34 (94%)

100 mg. complete relief occurred after 63 (75%) out of 84 injections. The value of P for the standard error of the difference between these two percentages is <0.00001.

A similar comparison of the effects of 100 mg. of pethidine hydrochloride with 50 and 100 mg. of 1C50 gave values of $P=0.26$ and <0.001 respectively. Comparison of 100 mg. of 1C50 with 10 mg. of morphine sulphate gave $P=0.38$.

Thus, the following equianalgesic doses may be suggested from these findings:

50 mg. 1C50 $\equiv$ 100 mg. pethidine hydrochloride
100 mg. 1C50 $\equiv$ 10 mg. morphine sulphate

The figures in the column headed "satisfactory relief" show similar comparative potencies for the different drugs.

Duration of Action

Table VIII shows the mean duration of action of the drugs when given in effective doses. Although 10 mg. of morphine sulphate and 100 mg. of 1C50 have the most prolonged actions, the differences between the various drugs are not statistically significant.

TABLE VIII

AVERAGE DURATION OF ACTION OF SOME ANALGESICS

Drug	Dose mg.	No. of Injections	Average Duration of Action
Morphine ..	10	22	5 hr. 24 min.
489C49	50	6	5 „
Pethidine ..	100	37	4 „ 42 „
268C49	75	12	4 „ 36 „
1C50	50	50	4 „ 24 „
	100	58	5 „ 42 „

TABLE IX

OCCURRENCE OF SIDE-EFFECTS IN PATIENTS

(The numerals in brackets at the head of each column indicate No. of injections)

Side-effect	Drugs and Doses					
	1C50 50 mg.	1C50 100 mg.	268C49 75 mg.	489C49 50 mg.	Morphine SO_4 10 mg.	Pethidine HCl 100 mg.
	(71)	(86)	(23)	(10)	(35)	(56)
Nausea	—	1	—	—	5	—
Vomiting ..	3	2	—	2	—	2
Vertigo	3	4	1	1	5	5
Drowsiness ..	9	3	2	1	5	5
Euphoria ..	1	—	—	4	—	1
Muscular weakness	—	—	—	2	—	—
Coloured urine ..	1	7	—	—	—	—

Effects on Respiratory Rate, Blood Pressure and Heart Rate

The effects of 1C50, morphine and pethidine on the rate of breathing, on blood pressure and on heart rate were slight and difficult to evaluate owing to the many variables which had to be taken into account.

Side Effects

The side effects noted during the clinical trial of the dithienylbutenylamine compounds are listed in Table XII. The following deserve comment:

Drowsiness and Sleep.—The occurrence of drowsiness and sleep is a valuable accompaniment of analgesia during the immediate post-operative period, and is, perhaps, hardly a side-effect. Table X shows the number of occasions when 1C50, pethidine and morphine produced satisfactory relief of pain with and without the simultaneous occurrence of sleep. Like the other two drugs, 1C50 has a true analgesic action but with the 100

TABLE X

NUMBER OF OCCASIONS ON WHICH ANALGESICS PRODUCED SATISFACTORY RELIEF OF PAIN WITH AND WITHOUT SLEEP

Drug and Dose	Relief With or Without Sleep	Relief Without Sleep
Compound 1C50:		
50 mg.	45	25
100 „	82	29
Pethidine HCl:		
100 mg.	42	22
Morphine SO_4:		
10 mg.	34	19

mg. dose the hypnotic effect predominates, and is greater than with 100 mg. of pethidine hydrochloride or 10 mg. of morphine sulphate.

It is, of course, a quite arbitrary choice to regard the production of sleep as complete relief of pain. However, since for our purposes the occurrence of painless oblivion is a useful therapeutic achievement we have felt justified in our choice. In any case, as may be seen in Table XI the number of occasions when sleep followed the administration

TABLE XI

TOTAL ADMINISTRATIONS OF VARIOUS ANALGESICS, BY NIGHT AND BY DAY, AFTER WHICH SLEEP OCCURRED

(Night, 9 p.m.–5 a.m.)

Drug and Dose	No. of Injections and when Administered	No. in which Sleep Occurred
1C50, 50 mg.	32 (day)	6
	39 (night)	26
100 „	38 (day)	14
	48 (night)	46
268C49, 75 mg.	17 (day)	3
	6 (night)	6
489C49, 50 mg.	8 (day)	2
	2 (night)	2
Pethidine HCl, 100 mg...	36 (day)	6
	20 (night)	16
Morphine SO_4, 10 mg. ..	19 (day)	5
	16 (night)	11

of the different drugs was much greater by night than by day. After 100 mg. of 1C50 sleep occurred on 46 out of 48 occasions at night, and only on 14 out of 38 occasions during the day.

Nausea and Vomiting.—These occurred very rarely and their relation to drug administration was difficult to evaluate.

Euphoria.—This was seldom seen in patients. It appeared to occur most frequently with 489C49.

Muscular Weakness.—We had previously noted in two normal subjects that 489C49 caused marked muscular weakness in doses of 12.5–25 mg. We were therefore prepared for this complication in our clinical trial and in fact we observed it in two out of eight patients who received this drug. The weakness was most marked in the arms and hands, there was great difficulty in speaking, and one of the patients had numbness of the cheeks and lips. Associated with this weakness there was marked sweating and flushing of the face and the physiological reactions were accompanied by considerable distress and restlessness. This most undesirable complication compelled us to abandon further use of 489C49. We did not observe this reaction with effective analgesic doses of 268C49 and 1C50.

Coloured Urine.—In one patient after 50 mg. and in seven patients after 100 mg. of 1C50 the urine passed several hours after injection was pink to purple in colour, becoming greenish-brown on standing. In one patient who received frequent injections of 100–150 mg. of this drug the pigments indigotin (blue) and indirubin (red) were identified and other yellow and yellow-green pigments were also presumed to be indigo derivatives. In all the patients who passed coloured urine, Obermayer's test for indican was positive. In this, ferric chloride and conc. HCl are added to the urine. $CHCl_3$ is then added, the urine is shaken and a blue colour develops in the $CHCl_3$ layer. However, the colour of the urine is not necessarily due to indigo derivatives, as the metabolic products of the drug are themselves coloured.

DISCUSSION

We propose to comment firstly on some of the methods which we have used in this study, and secondly on our assessment of the actions of the dithienylbutenylamine compounds.

Methods for Studying Analgesic Potency of the Drugs

The dithienyl compounds were given to us for studies in man after Adamson and Green (1950) had shown that these substances raised the pain threshold to heat and pressure stimuli in rats, rabbits and dogs (Green, 1953).

In previous work we had not been able to get consistent results when testing analgesic drugs with the radiant heat method of Hardy, Wolff, and Goodell (1940), so we made some preliminary observations with two methods in which the capacity of the drugs to relieve ischaemic muscle pain was studied.

(*a*) The method of Hewer and Keele (1948) was first used. The drugs under investigation are injected intravenously into one arm after ischaemic muscle pain of moderate intensity has been induced in the opposite forearm. The smallest dose of an analgesic drug which relieves this pain can be readily measured in this way, and it was found that the dithienyl compounds were intermediate in analgesic potency to morphine and pethidine (nearer the latter) (Keele, 1952). This agreed very well with the results in the dog (Green, 1953) and also with the clinical results reported in this paper.

(*b*) One of us has previously reported on the effectiveness of intramuscular injection of a dithienylbutenylamine compound (191C49, not discussed in the present paper) in increasing the number of ischaemic muscle contractions required to produce pain. This method, which was first

used by Harrison and Bigelow (1943), gives information concerning duration of action as well as analgesic potency.

However, we should like to emphasize that we do not attach too much importance to tests on analgesic drug action in experimental pain induced in normal persons. We agree with Denton and Beecher (1949) that the only satisfactory way to assess the analgesic capacity of a drug is to study its actions in patients with pathological pain, so that account can be taken of the complexity of the experience for which relief is desired. Hewer, Keele, Keele, and Nathan (1949) and Nathan (1952) have described a clinical method for studying analgesics in patients with chronic pain in whom subjective estimates of pain intensity are graded so as to provide quantitative data for the assessment of drug action. This method can be well controlled so that neither the patient—who usually keeps his own records—nor the person administering the drug, knows the nature or dose of the substance being given. Unfortunately, this procedure could not be used in the present study since the combined hypnotic properties of the dithienyl compounds made them more suitable for the relief of acute post-operative than of chronic pain. For this reason we had to modify the method of Hewer *et al.* (1949) and we came nearer to the procedure described by Keats, Beecher, and Mosteller (1950) for studying the effects of drugs on clinical pain.

There were, however, three respects in which our procedure differed from that of Keats *et al.* (1950):

(i) The dithienyl compounds, whose actions we were investigating for the first time in patients, had to be regarded as possibly toxic drugs. Indeed, we had already observed one very unpleasant side action—marked muscular weakness—with 489C49 in two normal subjects, and we had to be on the lookout for this and other toxic effects in patients. For this reason, we did not feel justified in using the dithienyl compounds as "unknowns" to the person who administered the drugs and observed the responses, though the nature of the injections was always unknown to the patient. We should, therefore, prefer to call our investigation a "pilot trial," though we think that the information we have obtained justifies certain definite conclusions concerning the therapeutic value of the drugs studied. It is, of course, easy to evaluate drugs which are either ineffective or toxic in therapeutic doses, and our method soon revealed the inadequate analgesic potency of 268C49 and the undesirable muscular weakness produced by 489C49. In comparing 1C50 with morphine and pethidine we have paid attention only to very clear-cut effects. For example, as shown in Table VII, we have taken as our significant end-point the complete relief of severe or very severe pain, and only the figures so obtained have been used to compare the effectiveness of the different drugs. Furthermore, in the statistical analysis of our results we have demanded more highly significant differences than are usually considered necessary; in no case have we regarded a difference as significant unless the value of P was <0.001.

(ii) We did not consider that it was justifiable to use injection of saline as a control, since Keats and Beecher (1950) have shown that this gives relief in only about 20% of patients. We feel that this proportion is too small for the carrying out of an extensive investigation in which the co-operation of both nursing staff and patients is required. We therefore decided to test the discriminatory power of our patients by administering two different doses (50 and 100 mg.) of 1C50, and Table VII shows the highly significant difference which was recorded between them ($P<0.00001$). Sometimes the 50 mg. dose gave no relief whereas 100 mg. was very effective; the absence of response to the smaller dose thus not only gave information which could have been obtained by injection of saline, but also indicated the lower limit of effective dosage of 1C50.

(iii) We have regarded sleep as equivalent to complete relief of pain, and we consider a combined hypnotic-analgesic drug very useful for post-operative pain. At night, when sleep is most necessary, the hypnotic action of all the drugs studied was much greater than by day (Table XI). We have not woken our patients to find out whether pain was still present, but we can get some idea of the analgesic, as distinct from the hypnotic, potency of the drugs from the number of occasions, chiefly by day, when pain was relieved without the patients going to sleep.

Effects of Dithienyl Compounds on Respiration

Owing to the many variables which influence respiratory rate during the post-operative period, we were unable to obtain reliable observations on patients, so the effects of the dithienyl compounds on breathing were studied in 12 normal subjects. Our results showed conclusively that pulmonary ventilation was reduced by these new compounds as much as by morphine and pethidine. Since the doses of the dithienyl compounds and of pethidine used in the normal subjects were no more than half those required therapeutically for relief of pain it can be said they were all at least as depressant to

respiration as morphine. Indeed 25 mg. of 1C50 appeared to be more depressant than 10 mg. of morphine, even though this represented only one quarter of the equianalgesic dose (i.e., 100 mg. of 1C50 is equianalgesic with 10 mg. of morphine). Our finding that pethidine is a powerful respiratory depressant is contrary to widely held opinions, but agrees fully with the results reported by Prescott, Ransom, Thorp, and Wilson (1949) and by Loeschke, Sweel, Kough, and Lambertsen (1953) who measured the respiratory depression in terms of a reduced response to the inhalation of CO_2-rich mixtures.

Assessment of the Dithienylbutenylamine Compounds

As a result of our studies on normal subjects and our pilot clinical trial in patients, we are now in a position to assess the status of the dithienyl compounds in comparison with morphine and pethidine.

Analgesic Potency.—The dithienyl compounds are somewhat more potent than pethidine and definitely less so than morphine. The most powerful member of the group, compound 1C50, is about twice as potent as pethidine and 1/10 as potent as morphine. These findings agree well with the results in the dog (Green, 1953) and with the observations reported by Keele (1952) for measurement of their analgesic potency against ischaemic muscular pain.

Hypnotic Action.—The dithienyl compounds have a greater hypnotic action than equianalgesic doses of morphine and pethidine. With 1C50 this additional effect was therapeutically helpful to patients with post-operative pain.

Euphoria.—The occurrence of euphoria in some of our subjects suggests strongly that the dithienyl compounds are capable of causing addiction. This agrees with the finding of Isbell, Fraser, and Wikler (1953) that 1C50 was a complete substitute for morphine in morphine addicts. Compound 1C50 and, to a less extent, the other members of the group must be regarded as drugs of addiction.

Respiratory Depression.—Like morphine and other powerful analgesics the dithienyl compounds are strong respiratory depressants. It is strange that it has so far proved impossible to find an analgesic drug with a morphine-like action which does not depress respiration. We found that the respiratory depression produced by the dithienyl compounds was fully antagonized by N-allylnormorphine (nalorphine), and Isbell *et al.* (1953) have also noted that nalorphine produces an abstinence syndrome in subjects receiving 1C50. These antagonisms by nalorphine bring 1C50 into line with morphine and all other substances with a morphine-like action, and differentiate it from other central nervous depressants, such as the barbiturates, which are not antagonized by nalorphine.

Nausea and Vomiting.—The dithienyl compounds are much less liable to produce nausea and vomiting than morphine. This difference is most clearly seen in ambulant normal subjects, the occurrence of these symptoms being very infrequent with all the drugs in post-operative patients. None of the drugs appeared to affect the alimentary tract (or bladder) in our patients.

Muscular Weakness.—The profound muscular weakness produced by 489C49 in a few normal subjects and patients was sufficient to contraindicate further trials with this drug. The other dithienyl compounds have much less action of this kind in normal persons and no muscular weakness has been detected in patients given 1C50.

Coloured Urine.—After 100 mg. doses of the dithienyl compounds (particularly 1C50) a purple coloration of the urine is liable to occur. In a few instances indican has been detected in the urine, but the coloration might also be due to breakdown products of the drug. There is no evidence that this coloration is harmful.

Other Effects.—*Dizziness* occurs more frequently with the dithienyl compounds than with morphine and pethidine. The same is also true for visual symptoms such as *photophobia* and *mistiness of vision.* The *pupil* is slightly *constricted. Blood pressure* and *heart rate* are not significantly affected.

Conclusions

Compound 191C49, previously studied (Keele, 1952), is a moderately effective analgesic drug which is occasionally liable to produce muscular weakness.

Compound 268C49 is too weak an analgesic to justify an extended therapeutic trial.

Compound 489C49 is a very good analgesic drug but its liability to cause alarming muscular weakness contraindicates its further use.

Compound 1C50 is an effective analgesic-hypnotic drug. It is an addictive drug which is not clinically superior to morphine except that it is less liable to cause nausea and vomiting. It has not caused muscular weakness, but it is a strong

respiratory depressant and causes a purple coloration of the urine. It has the disadvantage that it is unstable in aqueous solution and must be freshly dissolved before injection.

Summary

1. The analgesic and other actions of the following compounds have been studied in normal subjects and in patients with post-operative pain:

3-diethylamino-1 : 1-di-α-thienyl-1-butene (191C49).
3-ethylmethylamino-1 : 1-di-α-thienyl-1-butene (1C50).
3-pyrrolidino-1 : 1-di-α-thienyl-1-butene (268C49).
3-dimethylamino-1 : 1-di-α-thienyl-n-butane (489C49).

2. In a pilot clinical trial on 200 patients all the compounds had analgesic actions when injected intramuscularly in doses of 50–100 mg. The most potent member of the group was 1C50, the weakest was 268C49. Compound 1C50 was about twice as potent as pethidine and 1/10 as potent as morphine in the relief of post-operative pain. The dithienyl compounds were not active by mouth.

3. Both in normal subjects and patients these compounds had greater hypnotic actions than equianalgesic doses of pethidine and morphine.

4. The dithienyl compounds produced euphoria in normal subjects, and must be regarded as potential drugs of addiction.

5. In normal subjects these drugs depressed respiration as much as morphine and pethidine, 1C50 being the most potent in this respect. Nalorphine antagonized this respiratory depression.

6. The dithienyl compounds were much less liable to cause nausea and vomiting than morphine.

7. Other side-effects included:

(i) Muscular weakness, which was greatest with 489C49; this symptom was not encountered during the clinical trial with 1C50.

(ii) Purple coloration of the urine, which was sometimes associated with the presence of indican. Breakdown products of the drugs are also coloured.

No significant side-effects on the circulation, the alimentary tract or the bladder were seen in the patients.

8. Compound 1C50 had the highest therapeutic ratio of the dithienyl compounds. In equianalgesic doses in post-operative patients it was preferable to pethidine, but not quite so satisfactory as morphine.

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One of us (P. F.) was research assistant in the Departments of Anaesthesia and Pharmacology at the Middlesex Hospital when this work was done.

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INFLUENCE OF CYSTEINAMINE ON TUBERCULIN SENSITIVITY IN GUINEA-PIGS

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As a result of a systematic search for a non-toxic substance protecting against the toxic effects of irradiation, Bacq and his colleagues focused their attention on cysteinamine $HS—CH_2—CH_2—NH_2$, the pharmacological actions of which have been recently reviewed (Bacq, Dechamps, Fischer, Herve, Le Bihan, Lecomte, Pirotte, and Payet, 1953). As might have been expected with an -SH compound (see, for example, Cronkite, Brecher, and Chapman, 1951 ; Ord and Stocken, 1953), mice were protected if the drug was injected immediately before, but not after, irradiation. Of greater significance, however, was the finding that if the liver was screened during irradiation, cysteinamine protected even when injected after irradiation. This delayed effect was the more remarkable because though initial damage was indistinguishable in cysteinamine treated and non-treated groups, resolution began three to four days later in cysteinamine treated but not in untreated animals. Moreover, cysteinamine relieved symptoms of irradiation sickness in man. This post-irradiation effect cannot be explained simply. Sulphydryl groups, though immediately effective (Cronkite *et al.*, 1951), do not show a delayed protection against irradiation. In addition, Bacq and his colleagues describe other properties of the drug that cannot be explained in terms of -SH activity ; cysteinamine exerts anti-mitotic activity on tissue cultures from chick embryos ; increased amounts of ascorbic acid and other reducing substances are present in plasma and urine of treated animals ; in addition, the Arthus phenomenon in rabbits, and tuberculin response in man, are inhibited by the drug (Bacq *et al.*, 1953).

Apparently an attempt was made to explain these rather contradictory findings by the hypothesis that cysteinamine behaves first like an -SH compound, and secondly as a "stressor agent" stimulating adrenocortical activity, for a series of experiments to test this hypothesis was carried out. Bacq and his colleagues (1953) found that, although the concentration of ascorbic acid in the adrenal cortex falls with cysteinamine treatment, this does not indicate increased adrenocortical activity, because levels of adrenocortical cholesterol, of corticoid excretion, and of circulating eosinophils stay constant.

However, even if cysteinamine does not increase adrenocortical activity, it might induce "cortisone-like" effects by acting as an anti-metabolite in an -SH system. It has been postulated that in the guinea-pig, cortisone interferes with the anabolism of protein resulting in a fall in tissue non-protein -SH, thus facilitating the oxidation of ascorbic to dehydroascorbic acid, the latter inhibiting phosphoglucomutase with the result that the concentration of glucose-1-phosphate in the tissues rises, this being associated with a diminished sensitivity to tuberculin (Cornforth and Long, 1953 ; Long and Spensley, 1954 ; Long, 1954). The initial step in this hypothetical chain of events is a decrease in tissue non-protein -SH. If the reaction involving -SH groups were blocked by cysteinamine or by one of its metabolites, the final effect might resemble that induced by cortisone.

The experiments described in this paper support this hypothesis. Cysteinamine has an immediate effect—like the -SH compound reduced glutathione—in preventing desensitization by cortisone. This effect can be attributed to its -SH group. It is followed by a delayed reverse effect, for cysteinamine injected six hours before the tuberculin depresses sensitivity. This action, like that of cortisone, is antagonized by reduced glutathione and does not occur in ascorbic acid deficient guinea-pigs (Cornforth and Long, 1953) ; at this stage cysteinamine neither assists nor antagonizes the desensitizing action of cortisone.

METHODS

Albino guinea-pigs of the Hampstead strain, weighing 350–550 g. and fed on a pelleted diet (Bruce and Parkes, 1947), supplemented (except in animals maintained in a state of ascorbic acid deficiency) with unlimited cabbage, were injected with B.C.G. vaccine, and sensitivity to tuberculin was estimated by the

method of Long and Miles (1950). Groups of 10 guinea-pigs were used in all experiments.

Reduced glutathione was dissolved in gas-free distilled water and adjusted with sodium bicarbonate solution to *p*H 6 ; approximately 150 mg./kg. body weight in a volume of 2 ml. was injected intraperitoneally two hours before the tuberculin. Cortisone acetate (5 mg./kg.) was suspended in 1 ml. of normal saline and injected six hours before the tuberculin. Cysteinamine (100 mg./kg.) adjusted to *p*H 7.5 with hydrochloric acid (Becaptan) was injected intraperitoneally at the time intervals indicated in each experiment.

As in previous work, the diameter of the tuberculin lesions, after 24 hr., was found to be proportional to the logarithm of the dose of tuberculin ; and it was possible to estimate the degree of sensitivity by the relative positions of the dosage–response lines fitted to the mean lesion-diameters plotted against the logarithm of the dose. Furthermore, an analysis of variance was calculated and showed the significance of effects. All differences quoted have been tested in this way and found to be significant at the $P = 0.001$ level.

Results

Cysteinamine-Cortisone Relationships

Cortisone depressed sensitivity to tuberculin ; cysteinamine injected at the same time as the tuberculin did not influence sensitivity, but in cortisone-treated guinea-pigs it prevented desensitization

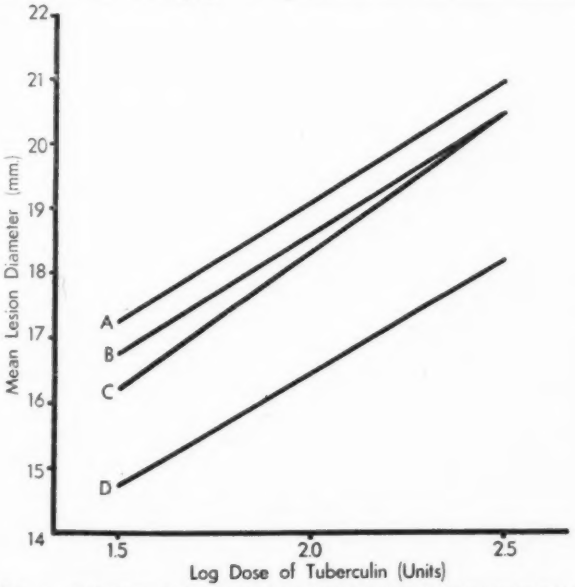

FIG. 1.—Response to 32 and 320 i.u. (International Units) tuberculin in sensitive guinea-pigs. A, Controls. B, Cysteinamine injected at same time as tuberculin. C, Cysteinamine injected at same time as tuberculin + cortisone injected six hours beforehand. D, Cortisone injected six hours before tuberculin.

(Fig. 1). Cortisone and cysteinamine both depressed sensitivity when injected six hours before the tuberculin, and this was true whether they were injected alone or together (Fig. 2).

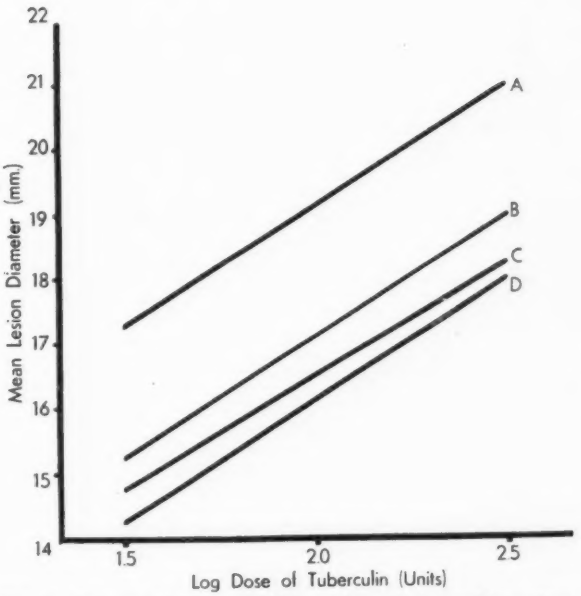

FIG. 2.—Response to 32 and 320 i.u. tuberculin in sensitive guinea-pigs. A, Controls. B, Cysteinamine injected six hours before the tuberculin. C, Cortisone injected six hours before the tuberculin. D, Cortisone + cysteinamine both injected six hours before the tuberculin.

Cysteinamine-Glutathione-Ascorbic Acid Relationships

Reduced glutathione injected at the same time as the tuberculin did not influence sensitivity, but prevented desensitization induced by cysteinamine injected six hours beforehand (Fig. 3). Cysteinamine did not influence sensitivity in ascorbic acid deficient guinea-pigs (Fig. 4).

Discussion

Cysteinamine, in the tuberculin sensitive guinea-pig, behaves first like an -SH compound ; this effect is in keeping with its protective power in mice when injected immediately before irradiation (Bacq *et al.*, 1953). This brief action is succeeded by a cortisone-like effect on sensitivity to tuberculin, which is antagonized by an -SH compound and does not occur in ascorbic acid deficient guinea-pigs (cf. Cornforth and Long, 1953)—an effect in keeping with the anti-mitotic and anti-allergic activity of the drug (Bacq *et al.*, 1953). This cortisone-like action, as already stated, cannot be attributed to increased adrenocortical activity (Bacq *et al.*, 1953). It is suggested that cysteinamine blocks the metabolism of -SH compounds after the manner of an anti-metabolite, and, as a result, the level of ascorbic acid and of other reducing substances in plasma and urine increases as described by Bacq. Further support for this hypothesis is obtained from the fact that

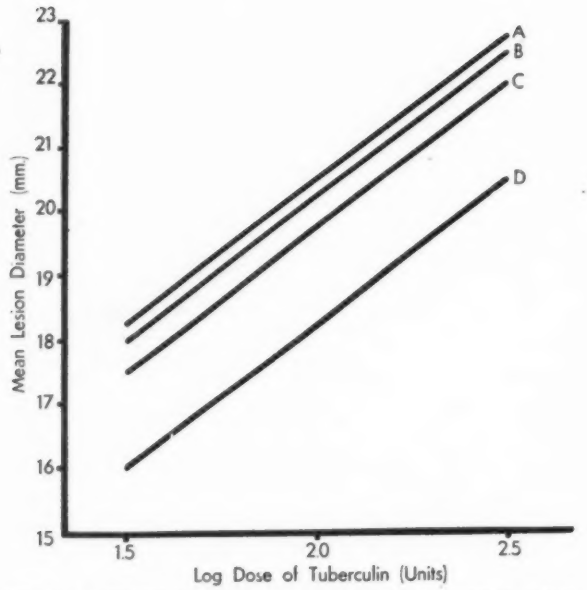

FIG. 3. — Response to 32 and 320 i.u. tuberculin in sensitive guinea-pigs. A, Reduced glutathione. B, Controls. C, Cysteinamine + reduced glutathione. D, Cysteinamine.

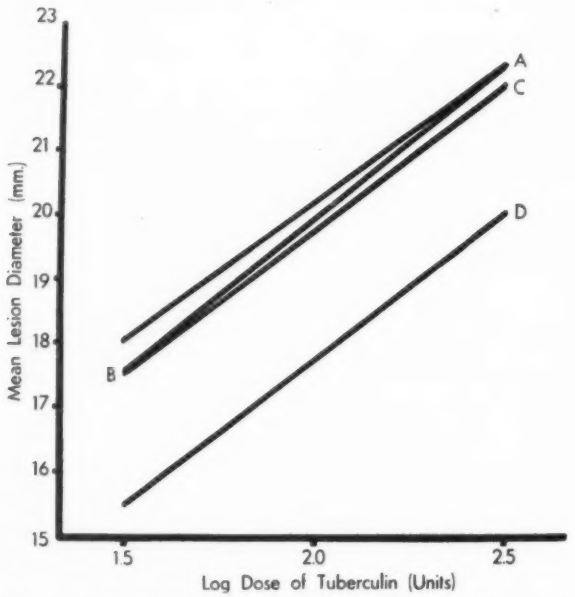

FIG. 4. — Response to 32 and 320 i.u. tuberculin in sensitive guinea-pigs. A, Cysteinamine. B, Ascorbic acid (provided as cabbage). C, Controls. D, Cysteinamine + ascorbic acid (provided as cabbage).

cortisone-induced hepatic hyperactivity is associated in many different species, including the guinea-pig, with hypertrophy of the liver (Lloyd and Long, 1953); whereas, in direct contrast, treatment with cysteinamine causes atrophy of the liver (Bacq *et al.*, 1953). Atrophy is commonly attributed to decreased activity, which, according to the hypothesis now advanced, can be attributed to a block of metabolism.

SUMMARY

1. Using the response to intradermal tuberculin as a measure of allergic hypersensitivity in albino guinea-pigs injected with B.C.G., it was found that:

(*a*) A single intraperitoneal injection of cysteinamine, given at the same time as the tuberculin, prevented desensitization by cortisone.

(*b*) A single intraperitoneal injection of cysteinamine, given six hours before the tuberculin, depressed sensitivity to tuberculin, and this action occurred in the presence or absence of cortisone.

(*c*) Desensitization produced by a single intraperitoneal injection of cysteinamine was prevented by reduced glutathione.

(*d*) Desensitization produced by a single injection of cysteinamine did not occur in ascorbic acid deficient guinea-pigs.

2. It is concluded that cysteinamine has two actions in the guinea-pig, an immediate action in which it behaves like an -SH compound, followed by a more prolonged action, in which it produces effects analogous to those of cortisone. It is suggested that the latter effects may be due to cysteinamine or one of its breakdown products behaving like an anti-metabolite.

I am grateful to Professor Z. M. Bacq for drawing my attention to the action of this interesting drug and for providing me with an advance copy of his recent review before publication.

I am indebted to Dr. Knud Tolderlund of the State Serum Institute, Copenhagen, for the B.C.G.; to Dr. R. H. W. Britton of Horlicks Ltd., Slough, for the Becaptan (cysteinamine); and to Miss M. V. Mussett for statistical analyses. It is a pleasure to acknowledge the continued advice of Dr. J. W. Cornforth, F.R.S., in this, as in other work relating to biochemical aspects of sensitivity to bacterial allergens.

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Brit. J. Pharmacol. (1954), **9**, 121.

THE GLYCOGENOLYTIC ACTION OF SODIUM SALICYLATE

BY

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Salicylate, in the form of salts of salicylic acid and of its acetyl derivative, has been used in the treatment of rheumatic fever for many years, and in one form or another it is probably the most widely used of household remedies ; yet our knowledge of its pharmacology remains deficient. This is not from lack of interest in the salicylates, for Gross and Greenberg (1948) have recently listed over 4,000 relevant publications. However, the literature does not afford a clear conception of the relation between the numerous established actions of the drug ; it is not even certain which actions are of therapeutic importance and which are purely side-effects. It seemed worth while, therefore, to look into some of the less obvious actions of salicylate. Of these perhaps the most striking is that observed by Lutwak-Mann (1942), that the administration of sodium salicylate caused almost complete disappearance of liver glycogen in the rat ; she concluded that this was evidence of interference with enzymatic processes by salicylate.

Such an action might be of therapeutic importance, but it would be hazardous to draw this inference from Lutwak-Mann's (1942) data, because large doses of salicylate were used, and because the mortality resulting from such doses made it impracticable to employ fasted animals, so that only fed rats with initially high liver glycogen concentrations were studied.

The primary aim of the present investigation was to re-examine the effect of salicylate on liver glycogen, to see whether the effect occurred at dosage levels more comparable with those used therapeutically, and to determine if it occurred in fasted as well as in recently fed animals.

The blood sugar was also estimated, since hyperglycaemia might accompany the expected glycogenolysis, although no related change in blood sugar concentration was found by Lutwak-Mann (1942). However, it had previously been reported that salicylate caused hyperglycaemia in the dog (Barbour and Herrmann, 1921) and in man (Morris and Graham, 1931).

METHODS

Swiss albino mice aged 3½ to 5 months were used. Glycogen was estimated, after hydrolysis by the method of Good *et al.* (1933), by ceric sulphate titration (Miller and van Slyke, 1936) ; blood sugar was determined by the method of Miller and van Slyke (1936).

The mice were dealt with in batches of 9–12 of the same sex per day ; two days before use they were given white bread in addition to the usual diet of rat cake. Two series of experiments were performed: in the first food was given until the beginning of the experiment ; in the second all food was withdrawn 18 hr. previously.

The mice were paired off for weight. One of each pair was given an intraperitoneal injection of 10 mg. sodium salicylate in 0.05 ml. water ; the other, control, animal received an intraperitoneal injection of 0.05 ml. normal saline. They were killed by a blow on the head at 1, 2, or 4 hr. after injection, and in each series an untreated group was killed at zero hr. 0.2 ml. blood for the estimation of glucose was obtained, after decapitation, from the neck, and liver was removed for the determination of glycogen content.

RESULTS

The data are presented to show (i) whether the animals were fed or fasted before the experiment, (ii) their sex, (iii) the time after injection at which they were killed, and (iv) whether they were controls or were salicylate-treated. The results were analysed by application of the t-test to the differences between means. Non-significant values of P will be omitted, and although those between 0.05 and 0.02 will be given, it is recommended that the minimum acceptable level of significance be taken at $P=0.02$.

TABLE I

MEAN LIVER GLYCOGEN CONCENTRATIONS OF FED AND OF FASTED SALICYLATE-TREATED AND CONTROL MICE, KILLED AT DIFFERENT TIMES AFTER INJECTION

The number of observations in each group is shown in parenthesis after each mean. P=0·05 is regarded as not significant.

Sex	Time Killed, (Hr. after Injection)	Fed			Fasted		
		Mean Liver Glycogen Concentration, mg./g.		P	Mean Liver Glycogen Concentration, mg./g.		P
		Control	Treated		Control	Treated	
Male	0	30·7 (16)	—		5·9 (20)	—	
	1	13·9 (15)	8·7 (15)	0·05	5·7 (20)	3·0 (20)	0·001
	2	13·9 (15)	5·2 (15)	0·01	3·8 (19)	2·3 (19)	0·02
	4	6·9 (14)	3·7 (14)	—	4·4 (19)	2·7 (19)	0·02
Female	0	39·8 (16)	—		8·1 (20)	—	
	1	26·4 (15)	12·9 (15)	0·001	8·5 (18)	4·4 (18)	0·001
	2	25·1 (14)	13·0 (14)	0·01	5·3 (20)	3·2 (20)	0·02
	4	8·3 (14)	6·6 (14)	—	5·7 (20)	4·8 (20)	—

Liver Glycogen

The liver glycogen results are presented in Table I. In both fed and fasted series the mean liver glycogen concentrations were significantly higher in the control females than in the control males. Thus, for example, in the fed series the male mean liver glycogen concentration at 1 hr. is 12.5 mg./g. less than the female ($0.01 > P > 0.001$).

The controls which had been fed till the beginning of the experiment show, in both sexes, sharp falls in mean liver glycogen concentration from 0 to 1 hr. These did not continue from 1 to 2 hr., but were followed by further falls from 2 to 4 hr. Such features may be interpreted as the result of the withdrawal of food, although possibly the fall from 0 to 1 hr. in the males is to a greater extent a result of the saline injection, since the male mice were more excitable than the females. In contrast, the salicylate-treated animals of both sexes had even lower mean liver glycogen concentrations at 1 hr., from which there was no rise within the period of the experiment. The differences between the saline and salicylate-treated animals' mean liver glycogen concentrations are significant at 2 hr. in both sexes, and at 1 hr. in the females.

The control mean liver glycogen concentrations of the animals fasted before the experiment gradually fell from 0 to 4 hr. The fasted salicylate-treated groups show sharp falls, in both sexes, from 0 to 1 hr., but not thereafter. The differences between the saline- and salicylate-treated groups' means were maximal at 1 hr. in both sexes, and although these differences were less than in the previous series, they are found to be highly significant on application of the t-test.

In summary, the mean liver glycogen concentrations of the salicylate-treated animals were significantly lower than those of the saline-treated controls, irrespective of sex, or whether fed or fasted immediately before the experiment.

Blood Sugar

The difference between the sexes was not marked in the control blood sugar concentrations, presented in Table II, although, in keeping with

TABLE II

MEAN BLOOD SUGAR CONCENTRATIONS OF FED AND OF FASTED SALICYLATE-TREATED AND CONTROL MICE, KILLED AT DIFFERENT TIMES AFTER INJECTION

The number of observations in each group is given in parenthesis after each mean. P=0·05 is regarded as not significant

Sex	Time Killed (Hr. after Injection)	Fed			Fasted		
		Mean Blood Sugar Concentration, mg./100 ml.		P	Mean Blood Sugar Concentration, mg./100 ml.		P
		Control	Treated		Control	Treated	
Male	0	167·8 (9)	—		114·9 (20)	—	
	1	145·5 (15)	150·9 (15)	—	108·6 (20)	118·2 (20)	—
	2	142·3 (13)	138·7 (13)	—	111·6 (18)	107·7 (18)	—
	4	108·2 (13)	87·3(13)	0·05	91·5 (16)	92·9 (16)	—
Female	0	153·5 (15)	—		99·7 (20)	—	
	1	130·4 (14)	162·1 (14)	0·01	105·3 (18)	148·0 (18)	0·001
	2	138·4 (11)	151·3 (11)	—	92·1 (20)	120·0 (20)	0·001
	4	114·8 (12)	108·3 (12)	—	91·1 (16)	102·4 (16)	—

their greater excitability, higher initial values were found in the males.

In both fed and fasted animals the mean blood sugar concentrations of the salicylate-treated males were, in general, almost identical with the corresponding control values ; no significant differences were found. It should be emphasized that these results do not prove that salicylate had no effect on the blood sugar level of the male mice ; but it is obvious that any significant effect with the doses used, and demonstrable by the use of substantially more animals, would be relatively small.

The salicylate-treated females had significantly higher mean blood sugar concentrations than those of the corresponding controls at 1 hr.; only in the fasted series was this so at 2 hr. The differences were greater and more significant in the fasted series.

To summarize: a significant hyperglycaemia occurred in the salicylate-treated females, whether fed or fasted immediately before the experiment ; but there was no significant change in blood sugar concentration in the male mice.

The sex difference in the response of the blood sugar concentration to sodium salicylate was not absolute. The disparity between the sexes lies not in the presence or absence of a certain effect, but in the relative extent of the effect. The sex difference in body weight, and therefore in dose expressed in relation to weight, may have been a contributory factor ; but by itself this cannot account for the discrepancy, because the mean doses of the fasted males and fed females were almost identical: the mean doses, in mg. sodium salicylate per g. body weight, were: fed males, 0.32 ; fed females, 0.36 ; fasted males, 0.35 ; fasted females, 0.40.

The explanation of this sex difference is not obvious. So many factors influence the blood sugar concentration that the possibilities are numerous. One factor is taken into account in the present investigation—the rate of reduction of liver glycogen concentration ; this did not differ greatly between the sexes. There may well be a sex difference in the rate of metabolism generally, and of the rate utilization of glucose in particular. This possibility merits further investigation.

Discussion

It may be concluded from the present data that sodium salicylate, in single non-toxic doses, caused a reduction of the liver glycogen concentration in young Swiss albino mice, irrespective of sex, or whether fed or fasted immediately before the experiment. This effect was a rapid one, occurring within 1–2 hr. of administration. It should be emphasized that no deaths resulted from the doses used, and that the salicylate-treated animals were indistinguishable in their behaviour from the saline-treated controls. Certain differences in detail between the present results and those of Lutwak-Mann (1942) will be noted ; the latter showed a more marked effect, justifying the use of the term " depletion " of liver glycogen ; this may have been due to the larger doses used. A more interesting difference is that Lutwak-Mann (1942) recorded the effect of sodium salicylate on rats' liver glycogen as being maximal 4 hr. after injection ; her fatalities also occurred at this time. In keeping with the present results, mice succumb to fatal doses of sodium salicylate within about one hour.

That single doses of sodium salicylate did or did not cause a significant hyperglycaemia, depending on sex, is very striking. The importance of this observation is that it strongly suggests that changes in blood sugar level due to salicylate should be regarded as secondary, i.e., as a by-product of the glycogenolysis and other, undefined, effects. It cannot be argued that breakdown of liver glycogen to glucose is sufficient to explain the results, since in the males the reduction in liver glycogen concentration is unaccounted for in the absence of definite hyperglycaemia.

It is tempting to speculate on the mechanism of the glycogenolysis. It may result from the inhibition of an enzyme concerned in the synthesis of glycogen, as Lutwak-Mann (1942) infers. However, the evidence seems insufficient to warrant such a far-reaching conclusion ; it remains to be shown that the glycogenolytic effect of sodium salicylate is due to a direct action of the drug on the liver. Furthermore, an acceptable hypothesis should take into reckoning the sex difference in the associated hyperglycaemia. This raises the question of whether the glycogenolytic action of salicylate may legitimately be considered as an isolated phenomenon. Such an assumption may be inadvisable.

Salicylate has been shown to have profound effects on the carbohydrate metabolism of mice. This does not necessarily apply to man. However, the finding of Morris and Graham (1931), that therapeutic doses of salicylate cause hyperglycaemia in man, suggests that salicylate may well have a glycogenolytic action in man.

Although the importance of the present findings is uncertain, it is clear that the classical concept of salicylate, as a drug whose main action is confined to the central nervous system, may be untenable.

Summary

1. The liver glycogen and blood sugar concentrations of young Swiss albino mice were determined 1, 2, and 4 hr. after the administration of 10 mg. sodium salicylate.

2. The liver glycogen concentrations of the salicylate-treated animals were significantly lower than those of saline-treated controls at 1 or 2 hr. after injection, irrespective of sex, or whether fed or fasted immediately before the experiment.

3. Though hyperglycaemia occurred in the salicylate-treated females, there was no significant change in blood sugar concentration in the males.

4. The results are briefly discussed, and it is concluded that the hyperglycaemia should be regarded as a secondary effect, and that salicylate probably has other major actions on carbohydrate metabolism.

The author wishes to thank Dr. G. M. Morant of the R.A.F. Institute of Aviation Medicine for his criticism of the statistics.

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BIOLOGICAL STANDARDIZATION OF "MSb POLYMER" (SODIUM *p*-MELAMINYL PHENYL STIBONATE)

BY

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The polymer of sodium *p*-melaminyl phenyl stibonate, referred to by Friedheim (1948) as "MSb," was first described by Friedheim and Berman (1946). They observed its pronounced prophylactic effect against *Trypanosoma equiperdum* infections of the mouse, and this was confirmed for *T. rhodesiense* in mice by Rollo, Williamson, and Lourie (1949) and for *T. gambiense* by Le Rouzic (1949a). Williamson (1949) correlated the period of prophylaxis against *T. rhodesiense*, after a single injection of MSb, with the amount of antimony in the viscera. Rollo *et al.* (1949) found that the drug possessed a higher therapeutic ratio than tryparsamide in laboratory infections, and Le Rouzic (1949b) found it possessed therapeutic activity against all stages of *T. gambiense* infections. However, the main virtue of this drug is its prolonged prophylactic action which Rollo *et al.* (1949) found to extend for 41 weeks after a single injection to mice.

We first became interested in the biological standardization of MSb when it was required by the Colonial Office for clinical trial in Africa. Since the polymers are not a single chemical entity, and some variation can occur between batches, biological standardization was necessary. Because the chief use of the substance was for prophylactic treatment it was more appropriate to have a prophylactic than a curative laboratory test for trypanocidal potency. But tests involving a long delay are quite impracticable for routine purposes. Lourie (private communication) suggested an assay extending over a period of four weeks, and it was with this suggestion in mind that we elaborated a prophylactic test. In its final form, this is complete in only two weeks. We considered it advisable to standardize the product biologically as completely as possible, and consequently included toxicity and therapeutic determinations. In this paper the symbol MSb refers to the sodium salt of the polymer only and not to the monomer; the monomer is relatively inactive and possesses no prophylactic activity against *T. rhodesiense* in mice.

METHODS

Toxicity and Therapeutic Experiments.—Fawn mice, 13 to 15 g. body weight, from our inbred laboratory stock, which were deprived of food overnight and fed two hours before injection, were assigned at random to groups consisting of 10, or more usually 20, animals. In the toxicity experiments deaths were recorded for seven days after a single subcutaneous injection.

In the therapeutic experiments mice were infected intraperitoneally with *T. equiperdum*, and on the following day those showing an infection of 1 to 10 trypanosomes per high power microscope field were injected subcutaneously with the drug. Blood smears from the tail were examined on the fifth day, since the maximum response was found from preliminary experiments to occur then.

The graphical method of de Beer (1945) was used to evaluate the results in toxicity and therapeutic experiments.

Prophylactic Experiments.—Twenty fawn mice, of 15 to 20 g. body weight, were used on each dose of a three-dose experiment, each mouse receiving a single subcutaneous injection of the laboratory standard (sample 1) of MSb. One week later, each mouse was infected intraperitoneally with 0.2 ml. of a suspension of *T. equiperdum*, which contained approximately 40,000 trypanosomes. A blood smear from the tail of each mouse was examined one week after infection and the number of mice free of trypanosomes was recorded. Similar groups were treated with pentamidine isethionate in order that a comparison could be made with a trypanocidal drug of well-defined chemical structure. The whole experiment was repeated until sufficient data for statistical analysis had been obtained.

Solutions.—The sodium salt of the polymer dissolved in water gave a cloudy solution. Since weak solutions were unstable and could not be prepared directly, a 6% w/v solution of MSb was prepared initially by mixing solid MSb and urea in the proportion of 3:4 and adding sufficient water at about 37° C. to give a clear solution; this solution was then diluted with sufficient water to give the desired concentration of MSb. As the solutions were relatively unstable, either precipitation or turbidity occurring after several days at room temperature, all solutions were used

within 20 min. of preparation, the toxicity and therapeutic tests being carried out simultaneously.

The arbitrary laboratory standard (sample 1) gave cloudy solutions and precipitated overnight at a concentration of 6% w/v, but at 3% w/v in 4% w/v aqueous urea the solution was quite clear; the other samples used gave clear solutions at 6% w/v and no precipitation occurred overnight.

Results

Toxicity Experiments

Most of the deaths, after a single subcutaneous injection of MSb, occurred within four days, although the animals were observed for seven days, and the mortalities at this time were used to assess the results. In order to cover the range of toxic doses at least four doses of both the laboratory standard and test sample were injected, using a dose ratio of 1.25. Usually 20 animals received each dose. The results are shown in Table I.

TABLE I

MSb POLYMER—ASSAY IN MICE OF SUBCUTANEOUS TOXICITY OF LABORATORY STANDARD (SAMPLE 1) AND OF UNKNOWN SAMPLES IN TERMS OF THIS STANDARD

Sample No.	Expt. No.	No. of Mice	LD50 (mg./kg.)	% Limits (P=0·05)	b	Potency	% Limits (P=0·05)
1	1	60	1,700	88·5–113·0	8·2		
	2	70	2,600	89·5–112·0	7·7		
	3	80	2,800	87·5–114·0	5·4		
	4	70	2,170	90·5–110·5	7·7		
	5	70	2,200	89·0–112·5	6·3		
	6	80	1,520	87·0–115·0	5·4		
	7	80	1,900	86·5–116·0	4·6		
	7*	80	2,040	92·0–109·0	8·7	1·10	86·0–116·0
2	3	100	2,510	91·0–110·0	6·7	1·11	85·5–117·0
3	4	90	2,790	88·5–113·0	5·6	0·81	86·0–116·5
4	5	90	2,580	90·0–111·5	6·3	0·86	85·0–117·5
5	6	100	2,120	83·5–119·5	3·4	0·66	80·5–124·0

* = Bulk standard as opposed to ampouled standard.

χ^2 tests for linearity of the regression lines (first approximation) and for homogeneity of the various estimates of slope were applied. These tests showed that the responses in any one regression line did not deviate significantly from linearity, and that the slopes of the regression lines obtained on various occasions with the laboratory standard and with the different test samples were homogeneous.

Therapeutic Experiments

Preliminary experiments, using a total of 280 mice, were carried out to investigate the effect of urea in the solutions against both *T. rhodesiense* and *T. equiperdum*. It was found that the addition of urea had no consistent effect upon the response of the trypanosomes. Urea was therefore used in all subsequent therapeutic tests as its presence was essential for the satisfactory preparation of the stronger solutions used in the toxicity experiments. The difference between the responses of the two species of trypanosome was just significant ($P \ll 0.05$). *T. equiperdum* was used in all later work because this organism has been extensively used for the biological standardization of organic arsenicals and trypanocides.

In the therapeutic experiments (3 + 3) dose assays were used with 20 infected animals on each dose. The results are shown in Table II. Again none of the regression lines deviated significantly ($P > 0.05$) from linearity; the various lines obtained with the standard formed a homogeneous series, as did those of the various test batches.

TABLE II

MSb POLYMER—ASSAY IN MICE OF THE EFFECTIVE POTENCY OF LABORATORY STANDARD (SAMPLE 1) AND OF UNKNOWN SAMPLES IN TERMS OF THIS STANDARD USING *T. EQUIPERDUM*. SAMPLES INJECTED SUBCUTANEOUSLY

Sample No.	Expt. No.	No. of Mice	ED50 (mg./kg.)	% Limits (P=0·05)	b	Potency	% Limits (P=0·05)
1	1	60	10·50	93·0–107·5	12·0		
	2	60	9·60	89·5–112·0	7·6		
	3	60	14·50	92·0–108·5	11·1		
	4	60	9·05	89·0–112·0	7·8		
	5	60	7·65	85·5–117·0	6·0		
	6	70	8·00	88·0–114·0	6·5		
	6*	70	8·00	88·0–114·0	6·5	1·00	83·5–120·0
2	2	60	8·60	88·5–113·0	7·6	1·12	84·5–118·5
3	3	60	14·30	92·5–108·0	11·7	1·01	89·0–112·0
4	4	60	10·25	92·0–108·5	11·0	0·93	87·5–114·0
5	5	60	10·35	93·0–107·5	12·5	0·92	87·0–115·0

ED50 = Dose which cleared trypanosomes from peripheral bloodstream in 50% of mice after five days.
* = Bulk standard as opposed to ampouled standard.

Prophylactic Experiments

The results of these experiments are shown in Table III. Untreated, infected control mice died within five days. Groups of animals were similarly tested with pentamidine isethionate, in order to test the method with a prophylactic drug of definite chemical structure.

The data from the five separate experiments (Table III) were adequate to warrant a thorough statistical analysis by the method of maximum

TABLE III

MSb POLYMER AND PENTAMIDINE ISETHIONATE. SUBCUTANEOUS PROPHYLACTIC EXPERIMENTS.
Mice infected with *T. equiperdum*

	MSb Polymer						Pentamidine Isethionate					
Dose (mg./kg.)	10·6	7·5	5·3				4·24	3·00	2·12			
Expt. No.	Mice Cleared of Trypanosomes on 7th Day/Mice Used			Slope (b)	PD50 (mg./kg.)	% Fiducial Limits (P=0·05	Mice Cleared of Trypanosomes on 7th Day/Mice Used			Slope (b)	PD50 (mg./kg.)	% Fiducial Limits (P=0·05)
1	17/20	12/20	6/16	4·49	6·36	59·3–119·8	14/20	13/20	9/20	2·15	2·27	—
2	16/20	8/20	2/20	7·06	8·09	88·3–115·5	—	—	—	—	—	
3	—	—	—	—	—		17/20	13/20	1/20	8·48	2·97	89·2–111·8
4	20/20	16/19	8/20	9·06	5·69	86·8–115·1	18/20	15/20	4/20	7·33	2·63	85·9–112·9
5	17/20	6/20	0/20	11·39	8·49	91·2–110·4	18/20	14/20	6/20	6·08	2·54	81·1–115·0
	b_c=7·06 Slopes $\chi^2_{[3]}$=6·739 (P=0·05–0·10)						b_c=5·49 Slopes $\chi^2_{[3]}$=10·148 (P=0·01–0·02) Omitting Expt. No. 1: b_c=7·22 Slopes $\chi^2_{[2]}$=1·007 (P=0·50–0·70)					

PD50 = median protective dose (see text).
Potency of MSb polymer in terms of pentamidine isethionate based on experiments 4 and 5 (maximum likelihood method):
Potency = 37.55%.
5% fiducial limits = 33.31-42.34%.

likelihood. In no experiment was there a significant curvilinear component in the regression line ($P>0.05$). But a χ^2 test on the variation in slope encountered with pentamidine isethionate showed a significant heterogeneity. This was attributable to the results in Experiment 1. Omitting this experiment the common slope was 7.22. None of the regression lines obtained with MSb showed a significant departure from rectilinearity ($P>0.05$), and the various estimates of slope were homogeneous. The common slope was 7.06. The potency of MSb in terms of pentamidine isethionate, based on experiments 4 and 5, was 37.55%, the fiducial limits ($P=0.05$) of this estimate being 33.31% to 42.34%.

DISCUSSION

In drafting biological standards for the quality control of samples of MSb, the following recommendations may be made:

Toxicity Test.—A toxicity test based on Gaddum's method (1943) could be used. The weighted arithmetic mean of the slope of the regression line is low ($b=5.83$), and a test using a dose ratio (S/U) of 1.25 with 20 animals on "standard" and "unknown" is fairly lenient (Table IV) although it would serve to eliminate any very toxic batches. On the other hand there are objections to this (1+1) dose assay (Perry, 1950), and it is better to use a (2+2) dose assay. A suitable dose ratio would be 1.8, and the results could be calculated by one of the recognized procedures.

Therapeutic Test.—For the therapeutic test, the (1+1) dose assay (Hawking, 1943) is slightly more stringent, because the common regression coefficient is higher ($b=8.58$). However, since the therapeutic test is just as, if not more, important than the toxicity test, a (2+2) dose assay should be adopted, with 20 animals receiving each dose. A suitable dose ratio would be 1.5 and the potency could be calculated by any suitable method.

TABLE IV

PERCENTAGE BATCHES WHICH WOULD FAIL A TOXICITY TEST IN A (1+1) DOSE ASSAY

n=20, b=5·83

S/U	Percentage Toxicity					
	100	110	120	130	140	150
	Percentage Failures					
1·15	16·5	34	53	70	83	91·5
1·20	12	26	45	62	77	87·5
1·25	8	19	35	53	69	81·5
1·30	5	13	27	44	60	74

Prophylactic Test.—A (2+2) dose assay for the prophylactic testing of MSb, using 20 animals on each dose, is adequate since the log. dose-probit regression line is rectilinear. A suitable dose ratio would be 1.8. The "PD50" would be defined as that dose which, when given subcutaneously one week before the intraperitoneal injection of a standard number of trypanosomes, prevented the appearance of trypanosomes in the peripheral blood in 50% of the mice for a further week; and the potency could be calculated by any suitable method.

Though Lourie suggested to us a prophylactic test extending over a period of four weeks, we have used one lasting only two weeks. Since neither procedure gives information on the duration of prophylaxis that might be expected, the shorter test possesses advantages as a routine assay.

Summary

1. The conditions for carrying out assays in mice for toxicity, therapeutic, and prophylactic potency against *T. equiperdum* using the polymer of sodium *p*-melaminyl phenyl stibonate (MSb) are described.

2. Statistical analysis showed that the regression lines in each of the three types of assay were without significant departure from rectilinearity, and the lines obtained in each type of test formed a homogeneous series.

3. For testing toxicity, a (1 + 1) dose assay could be used with a dose ratio (S/U) of 1.25; but a (2 + 2) dose assay is preferable, with a dose ratio of 1.8. For the therapeutic and prophylactic tests, a (2 + 2) dose assay ought to be employed, with dose ratios of 1.5 and 1.8 respectively. In each test 20 mice per dose should be used.

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